EXHIBIT 5

HUMAN GENE TRANSFER/THERAPY PROTOCOL

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Ex Vivo Gene Therapy of Familial Hypercholesterolemia

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I. Summary

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by a deficiency in the receptor that clears low density lipoprotein (LDL) from the serum (reviewed in Ref. 1 and 2). Patients with one abnormal LDL receptor allele have moderate elevations in plasma LDL and suffer premature coronary heart disease. Approximately 5% of all patients under 45 who have had a myocardial infarction carry this trait. Patients with two abnormal LDL receptor genes (homozygous deficient patients) have severe hypercholesterolemia and life-threatening coronary artery disease in childhood.

Strategies for treating patients with FH are directed at lowering the plasma level of LDL. In heterozygotes, this is accomplished through the administration of drugs that stimulate the expression of LDL receptor from the normal allele (2). This therapeutic approach is not effective in the treatment of homozygous deficient patients, especially those that retain <2% of residual LDL receptor activity. Partial amelioration of hyperlipidemia has been achieved in some homozygous deficient patients by diverting the portal circulation through a portacaval anastomosis (3) and by chronic plasmapheresis therapy (4). A more direct approach has been to correct the deficiency of hepatic LDL receptor by transplanting a liver that expresses normal levels of LDL receptor. Three patients that survived this procedure normalized their serum LDL-cholesterol (5-9).

We have used an authentic animal model for FH, the Watanabe Heritable Hyperlipidemic rabbit (WHHL), to develop gene therapies for the homozygous form of FH (10-13). The WHHL rabbit has a mutation in its LDL receptor gene which renders the receptor completely dysfunctional (12) leading to severe hypercholesterolemia, diffuse atherosclerosis, and premature death. The potential efficacy of gene therapy for FH is supported by a series of studies we have performed in the WHHL rabbit in which we have achieved metabolic improvement (14-18). Liver tissue was removed from WHHL rabbits and used to isolate hepatocytes and establish primary cultures. A functional rabbit LDL receptor gene was transduced into a high proportion of hepatocytes using recombinant retroviruses, and the genetically corrected cells were transplanted into the animal from which they were derived. Transplantation of the genetically corrected, autologous hepatocytes was associated with a 30-40% decrease in serum cholesterol that persisted for the duration of the experiment (4 months, Ref. 18). Recombinant derived LDL receptor RNA was detected in liver for at least 6 months. There was no apparent immunological response to the recombinant derived LDL receptor (18).

Another study of relevance to the proposal involves the isolation and retrovirus mediated gene transfer of human hepatocytes (19). We have successfully isolated viable human hepatocytes from 4 donors and have achieved gene transfer in a large proportion of cells. Hepatocytes transduced with a gene encoding LDL receptor express levels of functional protein that exceed normal endogenous levels.

Based on our preclinical studies, we propose a protocol to treat homozygous FH patients by *ex vivo* gene therapy. The patient population we plan to treat are FH homozygotes with symptomatic CAD who have a relatively poor prognosis but can tolerate a noncardiac surgical procedure with acceptable risks. Both children and adults will be eligible for this therapy. Patients will be evaluated over a six week period to determine their eligibility in the study and to establish metabolic baselines. The proposed therapy will be an adjunct to the more traditional therapies such as plasma exchange and drugs which will be reinstituted 6 weeks after gene therapy. Eligible patients will be admitted to the hospital and subjected to a two step procedure in which a portion of liver is removed on day 0 and hepatocytes are isolated and plated in culture. Recombinant retroviruses will be used to transduce a normal LDL receptor gene into

the cultured hepatocytes which will be harvested on day 3 and infused into the portal circulation of the patient through an indwelling catheter.

The patient will be evaluated for engraftment of corrected hepatocytes through a series of metabolic studies. Three months after gene therapy, a small amount of liver tissue will be harvested by percutaneous biopsy and analyzed for the presence of recombinant derived RNA and DNA.

II. Background

A. Gene Therapy

Monumental progress in the areas of molecular biology, virology, gene transfer technology, and human genome mapping are providing the foundation for a new era of biomedical research called somatic cell gene therapy (20). This innovative approach to therapeutics has the potential to prevent, treat, or potentially cure a variety of inherited and acquired diseases.

Two general strategies are being developed for the treatment of diseases based on somatic gene therapy. The most popular approach, which has been used in the first human trials, is referred to as *ex vivo* gene therapy. This is a multistep therapy which involves transplantation of genetically modified autologous cells. The specific steps are summarized below. Tissue that contains the appropriate target cell is harvested from the patient and the constituent cells are isolated, plated in culture, and exposed to recombinant retroviruses which are capable of transducing therapeutic genes into a significant proportion of the cells. The genetically modified cells are harvested and transplanted into the patient from which they were derived. An advantage of this approach is that gene transfer can be accomplished in an efficient and controlled manner *in vitro* and the genetically modified cells can be characterized prior to transplantation. *Ex vivo* gene therapy has several limitations. It is generally an invasive therapy requiring two surgical procedures (i.e., tissue harvest and cell transplantation). In addition, the efficacy of *ex vivo* gene therapy will necessarily be dependent upon the capacity and performance of the cell transplantation, which in some systems can be limiting.

An alternative and potentially more effective approach to gene therapy is direct delivery of the therapeutic gene to cells *in vivo*. This would require the development of gene transfer substrates capable of targeting to the appropriate cell and internalizing the gene so that it is transported to the nucleus and expressed. Techniques of *in vivo* gene transfer are very early in development.

The development of a specific gene therapy protocol is a complex process that requires an understanding of the pathophysiology of the disease as well as an appreciation of the available technology. A variety of somatic cells have been considered potential targets for somatic gene transfer including hepatocytes (21), hematopoietic stem cells (22), respiratory airway epithelial cells (23), lymphocytes (24), endothelial cells (25), skeletal (26) and cardiac myocytes (27), fibroblasts (28), keratinocytes (29), and cells of the central nervous system (30).

An equally wide array of gene transfer substrates are available. Recombinant retroviruses are the preferred substrate for transducing genes into dividing cells *in vitro* (31). Approaches for *in vivo* gene transfer are early in development and include viral [adenoviruses (23), adeno-associated viruses (32), herpes simplex virus (30)] and nonviral substrates [liposomes (33,34), and DNA/protein complexes (35)].

We are interested in developing therapies for the treatment of metabolic disorders. The model we have used is familial hypercholesterolemia (FH) which is caused by defects in the receptor that binds low density lipoproteins (LDL).

B. Familial Hypercholesterolemia

1. Genetics

FH is an autosomal dominant disorder caused by abnormalities in the function or expression of LDL receptors (1,2). Patients who inherit one abnormal allele have moderate elevations in plasma LDL and suffer premature coronary heart disease (CAD). The prevalence of heterozygotes in most populations is 1 in 500 and they represent approximately 5% of all patients under 45 who have had a myocardial infarct. Patients with two abnormal LDL receptor alleles (homozygotes or compound heterozygotes) have severe hypercholesterolemia and life-threatening CAD. Features of FH that relate to the proposed *ex vivo* gene therapy will be summarized below. In subsequent discussions, FH will refer to the more severe clinical syndrome that is associated with two abnormal LDL receptor alleles.

The molecular basis of FH lies in the gene that encodes LDL receptors. Characterization of mutant alleles has revealed a variety of mutations including deletions, insertions, missense mutations, and nonsense mutations (2, 36). This genotypic heterogeneity leads to variable consequences in the biochemical function of the receptor which are classified in four general groups. Class I mutations are associated with no detectable protein and are often caused by gene deletions. Class 2 mutations lead to abnormalities in intracellular processing of the protein. Class 3 mutations specifically affect binding the ligand LDL, and class 4 mutations encode receptor proteins that do not cluster in coated pits.

2. Metabolism

An important issue in the development of gene therapy for FH relates to the specific somatic cell that is the target for gene transfer. We believe that the hepatocyte is the preferred target cell for gene therapy of FH. The rationale for this will be described in a broader discussion of the metabolic steps involved in the maintenance of cholesterol homeostasis *in vivo*.

Virtually all somatic cells contain a group of highly regulated metabolic pathways capable of stabilizing intracellular cholesterol concentrations (2). Cholesterol can be produced by de novo biosynthesis or can enter the hepatocyte by receptor-mediated and non-receptor mediated uptake of lipoproteins and lysosomal hydrolysis of the component cholesterol esters. The enzyme that catalyzes the rate limiting step in de novo biosynthesis, HMG CoA reductase, and the receptor most responsible for lipoprotein catabolism, the LDL receptor, are both subject to feedback inhibition by intracellular cholesterol. Cholesterol has limited metabolic fates in most non-hepatic cells; it can be incorporated into membranes or stored intracellularly in the form of cholesteryl esters. The enzyme responsible for esterification of cholesterol, acylCoA: cholesterol acyltransferase, is activated in the presence of excess intracellular cholesterol (37).

The liver modulates cholesterol homeostasis *in vivo* through a variety of metabolic functions that are uniquely expressed in its parenchymal cells. Hepatocytes secrete very low density lipoproteins (VLDL) which are the precursor to most other lipoproteins involved in endogenous metabolism of cholesterol (38). VLDL, a large triglyceride-rich lipoprotein, is converted to IDL through the action of lipoprotein lipase in capillary beds. IDL is recognized by the receptor for LDL through interactions with its two apoproteins, apo B100 and apo E. The

fraction of IDL that escapes receptor mediated uptake and degradation is converted to LDL in capillary beds (2). LDL is the primary carrier of cholesterol in the plasma and has been implicated in the development of atherosclerosis. Hepatic LDL receptors contribute to >90% of the high affinity uptake and degradation of LDL *in vivo* (39). A complete deficiency of LDL receptor activity in FH leads to a precarious metabolic state in which the catabolism of LDL and its precursor lipoprotein IDL is decreased. Diminished high affinity uptake of IDL leads to a marked overproduction of LDL, which in the setting of decreased LDL catabolism, results in massive hypercholesterolemia (11).

Hepatocytes along with absorptive epithelia of the intestine have the unique function of expressing apoproteins associated with the other major lipoprotein of the endogenous pathway, HDL. Liver and bowel directly secrete nascent HDL particles which are converted to mature HDL through the action of a variety of enzymes. HDL is a very dynamic lipoprotein whose specific functions in cholesterol homeostasis remain poorly defined, but is most often associated with a process termed reverse cholesterol transport or the return of cholesterol from peripheral cells to the liver for excretion into bile.

Liver is the only organ capable of excreting cholesterol from the body, a function which is critical to the maintenance of cholesterol balance *in vivo* (39). This is accomplished through the conversion of free cholesterol to bile acids and the formation of bile (composed of cholesterol and bile acids) which is secreted from hepatocytes and eventually excreted from the body. Biliary cholesterol is derived from a pool of metabolically active free cholesterol that is formed by de novo synthesis and receptor-mediated degradation of lipoproteins (40). In humans, over one gram of cholesterol is excreted per day by this route (39).

In summary, the liver is the primary organ responsible for regulation of cholesterol homeostasis, *in vivo*, and the receptor for LDL plays an important role in this regulation. Hepatocytes are the cells primarily responsible for catabolizing LDL and the only cell capable of excreting cholesterol. We therefore believe that the hepatocyte is the preferred target for gene transfer in gene replacement therapies of FH. The success of orthotopic liver transplantation in the treatment of FH provides compelling support for the hypothesis that expression of hepatic LDL receptor activity is sufficient for metabolic correction *in vivo* (see Section II.B.5). It is possible, but unlikely, that expression of LDL receptor in non-hepatic tissues could improve the hyperlipidemia in FH without peripheral adverse effects.

3. Clinical

A risk/benefit assessment of gene therapy for FH requires a thorough understanding of the natural history of the disease. In general, FH homozygotes have severe hypercholesterolemia from birth and develop peripheral stigmata of the disease by age 4 including cutaneous xanthomas, tendinous xanthomas, and arcus cornae (2). The most morbid and life limiting aspect of the disease relates to the development of a characteristic pattern of atherosclerosis. The coronary arteries and proximal aorta are severely involved while the cerebral arteries and distal aorta are relatively spared. Accelerated coronary atherosclerosis leads to a high incidence of myocardial infarction in children and teenagers. Disease in the ascending aorta led to a variety of complications, including narrowing of the coronary ostium and aortic stenosis due to valvular or supravalvular aortic disease. Patients usually die of sequelae of CAD before the age of 30.

More detailed characterizations of FH homozygotes have demonstrated clinical heterogeneity based on genotype specific variations in the natural history of the disease. The basic premise is that molecular heterogeneity leads to genotype specific variation in the level of residual LDL receptor function which may correlate with severity and progression of the

disease. Sprecher *et al.*, characterized the residual LDL receptor activity in fibroblasts of 14 FH patients and attempted to correlate this with several clinical indices (41). They demonstrated a statistically significant inverse correlation between residual LDL receptor activity and 1) pretreatment cholesterol and LDL levels, and 2) age of onset of angina pectoris. They also noted an association between LDL receptor activity and cholesterol reduction in response to conventional pharmacological therapy. In another study, Goldstein and Brown analyzed 57 homozygous deficient patients for residual LDL receptor activity in fibroblasts and classified them as receptor-negative (<2% of control, N=31) and receptor-defective (>2% of control, N=26) (42). Receptor status was correlated with age of onset and severity of coronary artery disease. Manifestations of coronary artery disease developed before the age of 10 in 32% (10/31) of receptor-negative patients and in only 4% (1/26) of the receptor-defective patients. Furthermore, 25% (8/31) of the receptor-negative patients died of the sequelae of coronary artery disease before the age of 25 (mean = 11 years old), whereas only 4% (1/26) of receptor-defective patients died during this time interval. This observation was confirmed in a study by Sprecher *et al.* that characterized a group of 16 unrelated FH homozygotes with respect to the presence or absence of overt CAD (43). Patients with symptomatic CAD had extremely high total serum cholesterols (862 +/- 62 mg/dl) and were primarily receptor negative. Four of seven symptomatic patients died of CAD; the average age death was 12 years of age. The asymptomatic group had lower serum cholesterols (629 +/- 75 mg/dl) and were primarily receptor defective. No deaths were reported in this group. Haitas *et al.* studied a population of White Afrikaners that have a high incidence of receptor defective FH (44). A group of 49 patients, symptomatic with CAD, had an average serum cholesterol equal to 744 mg/dl; and 11 patients eventually died at an average age of 18 years old. Additional studies in various ethnic groups including Lebanese (45), Japanese (46, 47), and French Canadians (48) confirm these findings. A table of the population studies reviewed above are summarized in Table 1.

TABLE 1

Reference	Ethnic Group	CAD	Receptor Status	Frequent Mutation	Avg. Serum Cholesterol mg/dl	Deaths #	Deaths Age
42	- - -	17/31	negative	- - -	- - -	8/31	11 yo
		11/26	defective	- - -	- - -	1/26	23 yo
43	- - -	7/7	negative *	- - -	862+/-63	4/7	12 yo
		0/9	defective *	- - -	626+/-75	0/9	- - -
44	White Afrikaner	48/49	defective	- - -	744	11/49	18 yo
45,49	Lebanese	- - -	- - -	nonsense	729	11/52	21 yo
48, 50	French Canadian	- - -	- - -	Deletions	557-1532	5/19	21 yo
47	Japanese	- - -	- - -	- - -	772	6/10	26 yo
46	Japanese	- - -	- - -	- - -	735	5/25	11 yo

* Patients were classified according to the presence or absence of CAD. 5/7 patients with CAD were receptor negative, 1/7 receptor defective and 1/7 had unknown receptor status. 6/9 without CAD were receptor defective, 1/9 was receptor negative, and 2/9 had unknown receptor status.

4. Level and Expression of Transferred Gene

The clinical correlations described in the previous section are of potential significance for gene therapy of FH. The ultimate goal of gene therapy in this disease is to replace the normal complement of LDL receptor activity in all hepatocytes thereby normalizing total serum cholesterol. This, however, will be a difficult task because of the large number of potential target cells in a human liver. The relevant question in terms of this protocol is whether partial reconstitution of LDL receptor function may be therapeutic. This issue will be further discussed in section IV.

Another issue in the development of gene therapy for FH is the importance of regulating the expression of the transgene. The normal endogenous LDL receptor plays an important role in the maintenance of cellular cholesterol balance. Excess accumulation of intracellular sterol leads to suppression of exogenous sterol uptake by direct repression of LDL receptor gene transcription (51, 52). Most LDL receptor gene transfer systems under consideration for gene therapy utilize transcriptional signals that would result in constitutive expression of the LDL receptor gene (14, 53). A potential problem with this approach is that the constant unregulated uptake of LDL could lead to excessive accumulation of intracellular cholesterol and cholesteryl esters which may be toxic to hepatocytes.

Previous LDL receptor gene transfer experiments have used constitutive or inducible promoters to drive the expression of the transferred gene. LDL receptor genes have been introduced into a variety of cell lines by transfection (54, 55) or retroviral infection (14, 53). Constitutive and high level expression of the LDL receptor transgene in these cell lines had no reported effect on cell growth or function. However, transfected cell lines may not be the best experimental system to test the effects of excessive accumulation of intracellular cholesterol because they have high requirements for exogenous cholesterol by virtue of their ongoing cell division. More recently, we have used recombinant retroviruses to transfer a functional human LDL receptor gene into hepatocytes from a rabbit that is genetically deficient in LDL receptor expression (see Section II.C.1, Ref. 14). Constitutive overexpression of LDL receptor (greater than 4-fold over normal levels) in these infected cells had no detectable effect on cell viability or morphology during the time course of these experiments (48 to 72 hours following the retroviral infection). More importantly, the cells persisted and continued to express high levels of the recombinant transcript after transplantation into autologous LDL receptor deficient rabbits (see below).

Another informative gene transfer experiment was recently described by Hofmann *et al.*, where a human LDL receptor gene, driven by the metallothionine promoter, was introduced into the germ line of a mouse (56, 57). Several transgenic animals that resulted from this experiment were shown to express high levels of human LDL receptor in liver when the transgene was induced with $CdSO_4$. Under these conditions, the transgenic animals demonstrated markedly increased catabolism of ^{125}I-labeled LDL and a virtual disappearance of serum LDL; no ontoward effects of this hepatic overexpression of LDL receptor were described (56). In addition, hepatic overexpression of the LDL receptor transgene prevented the development of hypercholesterolemia in response to a high lipid diet (57).

5. Alternative Therapies

A variety of surgical and pharmacologic therapies have been tried in the treatment of homozygous FH. These include diet, pharmacologic agents (58-63), portacaval shunts (3, 64, 65), plasma exchange (4, 66-74), LDL apheresis (75-77), and orthotopic liver transplantation (5-9). The response of FH homozygotes to drugs is dependent, in part, on the residual function of LDL receptor. Intensive pharmocologic and dietary therapy has been

uniformly unsuccessful in receptor negative homozygotes (<2 residual LDL receptor activity, Ref. 62). More encouraging results have been achieved in receptor defective patients (>2% residual LDL receptor activity) treated with a combination of nicotinic acid, a bile binder and an HMG CoA reductase inhibitor (61). An alternative pharmacologic approach to treatment of homozygous FH involves the use of the drug probucol (78). This lipophilic drug is an antioxidant that has been associated with regression of xanthomas in some patients (79). Its mechanism of action is unknown, however, data in the WHHL rabbit suggests that it may inhibit the formation of oxidized LDL and promote regression of xanthomas (80). Unfortunately, its beneficial effect on LDL metabolism may be counterbalanced by an associated decrease in HDL.

The most widely used form of therapy for homozygous FH involves repeated purging of LDL from the blood through the use of plasma exchange or LDL apheresis (4, 66-77). The longest clinical experience using this approach has been with plasma exchange. Patients treated every 1 to 2 weeks achieve substantial but variable decreases in serum cholesterol. Plasma exchange in combination with pharmacologic therapy can lead to a 50% reduction in serum cholesterol in some patients. A theoretical disadvantage of plasma exchange is that it causes a depletion of HDL. A protocol of plasma exchange every 1 to 2 weeks for periods greater than 3 years is associated with diminished progression and occasional regression of atherosclerosis in the coronary arteries and proximal aorta. This, however, is not always the case as exemplified by a patient who experienced progression of cardiovascular disease during chronic plasma exchange therapy (81).

Recent advances in this therapeutic concept, called LDL apheresis, have involved the development of extracorporial devices that selectively remove LDL (75-77). An advantage of this technique is that it does not perturb HDL levels. The long term consequences of LDL apheresis remains to be determined. Both plasma exchange and LDL apheresis are complicated by the necessity of weekly or bimonthly treatments for the life of the patient. No longitudinal studies have shown an improvement in the morbidity or morality of FH patients using these procedures.

Several surgical procedures have been attempted to affect more permanent improvements in FH homozygotes. One approach is to perform an ileal bypass to promote intestinal losses of bile and cholesterol. FH homozygotes usually do not respond to this procedure (82). Another approach is to surgically create a portacaval shunt (3, 64, 65). A review of the experience with portacaval shunts in 45 FH homozygotes described a 25% decline in serum cholesterol in 38% of the patients (3). Metabolic studies indicate this effect is due primarily to a decrease in LDL synthesis. The long term consequences of portacaval shunts are unclear.

The most dramatic and effective treatment of homozygous FH has been orthotopic liver transplantation with an organ that expresses normal levels of LDL receptor. To date, liver transplant has been attempted in 4 individuals, three of which were the recipients of a combined heart and liver transplant procedure (5-9). A summary of these patients are described below.

S.J. was a FH homozygote who underwent a combined heart/liver transplant in 1983 at the age of 6 (5,6). Her total serum cholesterol fell from 1100 mg/dl to 200-300 mg/dl following the transplant, and finally to 150-200 mg/dl with the addition of an HMG Co-A reductase inhibitor. She did well after the operation but eventually developed complications of immunosuppressive therapy and died in 1991. A combined liver/heart transplant was unsuccessfully attempted on a 17 year old girl with FH and end-stage cardiomyopathy (7). The patient died of multiple complications that occurred in the perioperative period. A third liver/heart transplant was attempted in a FH homozygote by a group in Spain (9). The patient survived the procedure and realized a 71% decline in total serum cholesterol postoperatively. The final case was a 12 year old boy with homozygous FH who underwent a liver transplant in

1986 (8). There was a striking reduction in total serum cholesterol (76% decrease) after the transplant. The experience with liver transplantation vividly illustrates the importance of hepatic LDL receptor expression in modulating *in vivo* cholesterol metabolism. The spectacular metabolic improvement obtained following liver transplantation must be balanced with the associated perioperative mortality and substantial long term morbidity secondary to chronic immunosuppression.

C. Preclinical Studies

Our preclinical studies have focused in on two areas. The majority of our work has utilized a rabbit animal model for FH. We have used this model to develop the technology of liver-directed gene therapies and to assess efficacy of LDL receptor gene transfer *in vivo*. We have also used liver tissue from donors for orthotopic liver transplantation to develop methods for isolating and transducing human hepatocytes.

1. *Ex Vivo* Gene Therapy in the Watanabe Heritable Hyperlipidemic Rabbit

The animal used in our preclinical studies, called the Watanabe Heritable Hyperlipidemic rabbit (WHHL), was described in the 1970's by Dr. Watanabe (10). This animal demonstrates the clinical and metabolic abnormalities similar to those associated with FH in humans . The WHHL rabbit was instrumental in delineating key steps in the regulation of cholesterol metabolism and has been very useful in the design and testing of gene therapies for FH (11,13).

The specific mutation responsible for the WHHL trait is an inframe deletion of 12 nucleotides in the ligand binding domain of the receptor (12). This mutation leads to expression of a dysfunctional receptor protein that is inefficiently processed and unable to bind ligand (12, 83). Quantitative LDL binding analyses of WHHL derived cells and tissues, and *in vivo* measurements of receptor dependent lipoprotein uptake, indicate that the receptor deficiency of this animal is essentially complete (<5% of control) (84-88). The metabolic consequences of this defect in rabbits closely resembles those described in patients with FH. In both cases, there is a selective accumulation of lipoproteins that contain apo B100, the polypeptide which is specifically recognized by the LDL receptor (i.e., VLDL, IDL and LDL). Lipoproteins not recognized by LDL receptor remain unchanged except for HDL which is slightly decreased in both WHHL rabbits and FH patients. The validity of the WHHL rabbit model was initially questioned when it was noted that the rabbits had elevated levels of both triglycerides and cholesterols, while FH patients had increased cholesterol but normal triglyceride. This was subsequently ascribed to unusually high triglyceride content in rabbit derived LDL (11).

The full spectrum of clinical manifestations described in FH homozygotes are observed in the WHHL rabbit. Peripheral manifestations of severe hypercholesterolemia in the WHHL rabbit include lesions of the paws which resemble tendinous xanthomas (10). WHHL rabbits, like FH homozygotes, develop severe atherosclerosis attributable to severely elevated LDL that is restricted to the coronary arteries and proximal aorta (10). We have used the WHHL rabbit as a model to develop *ex vivo* approaches to liver directed gene therapy.

The first step towards the development of these therapies was to design methods for isolating WHHL hepatocytes and efficiently transducing functional LDL receptor genes into the cells. A series of retroviral vectors that express the gene for human LDL receptor were constructed, each differing in the transcriptional elements used to drive LDL receptor expression (14). Helper-free amphotropic virus stocks representing each construct were then used to infect primary cultures of hepatocytes that were isolated from newborn WHHL rabbits.

The efficiency of transduction, as measured by Southern analysis of integrated proviral sequences, ranged from 20% to 100%. Expression of human LDL receptor was analyzed by blot hybridization analysis of total cellular RNA and by biochemical and *in situ* analyses of transduced cultures for receptor function. The vector in which the expression of LDL receptor was driven by the viral long terminal repeat sequence produced the greatest quantity of LDL receptor RNA and protein in WHHL hepatocytes; LDL receptor activity approached normal levels in these cultures.

The next and more difficult step was to develop methods for transplanting LDL receptor-expressing hepatocytes into WHHL rabbits in a way that the cells function and persist. Hepatocytes were isolated from an outbred strain of rabbits that express normal levels of LDL receptor (New Zealand white strain) and transplanted into WHHL recipients by direct injection into the portal vein or into the peritoneal cavity attached to microcarrier beads (17). Transplantation of NZW hepatocytes using either method led to a 25% decrease in total plasma cholesterol over a 3 to 4 day period with a gradual return to pretreatment levels after 10 days. The resulting decrease in total plasma cholesterol was due to coordinate decreases in lipoproteins that are known ligands for the LDL receptor. Transplantation of allogeneic WHHL hepatocytes into WHHL recipients did not lead to a decrease in total plasma cholesterol.

These experiments indicate that ectopically placed hepatocytes (in liver sinusoids or in the peritoneal cavity) can function with respect to lowering cholesterol, but only in a transient manner. Wiederkehr *et. al.* have demonstrated more prolonged function of transplanted NZW hepatocytes in WHHL rabbits when the animals were chronically immunosuppressed with cyclosporin (89). This suggests that the deterioration in LDL receptor function which occurred in our study was due to rejection of the cells. This could be due to major histocompatability complex (MHC) incompatabilities between the two strains of rabbits or an immunological response to the recombinant derived LDL receptor protein on the donor-derived cells.

The feasibility of *ex vivo* gene therapy in the WHHL rabbit was demonstrated in a modification of the allogeneic experiments described above (16). Liver was harvested from a WHHL rabbit and hepatocytes were harvested and plated in primary culture. Recombinant genes were transduced into 10-20 % of the cultured hepatocytes which were harvested 4 days after the initial plating and transplanted into a group of WHHL recipients. Our colony of WHHL rabbits are outbred so this would constitute an allogeneic transplant. There were two experimental groups: animals that received allogeneic WHHL hepatocytes that were either mock-infected (N=6) or infected with recombinant retroviruses that contain a functional human LDL receptor gene (N=7).

Analysis of the primary cultures prior to transplantation indicated that the bulk population of cells expressed levels of recombinant LDL receptor protein and RNA in excess of that found in NZW hepatocytes. The metabolic consequences of hepatocyte transplantation was assessed by measuring changes in total serum cholesterol. No significant change in serum cholesterol was noted in animals that received mock infected hepatocytes. However, a substantial (30-40%) but, again, transient decline in serum cholesterol was achieved after transplantation of the LDL receptor transduced cells.

Transplant recipients were further characterized with respect to recombinant gene transfer and expression. Liver tissue was harvested 10 minutes, 24 hours, and 19 days after transplantation of the LDL receptor transduced hepatocytes and analyzed for the presence of recombinant-derived RNA by RNase protection and proviral DNA by the polymerase chain reaction. Recombinant human LDL receptor RNA was detected 10 minutes and 24 hours after transplantation at 2-4 % the level of the endogenous transcript. Proviral DNA was also detected at both time points. Analysis of tissue harvested 19 days after transplantation detected neither the proviral DNA or the recombinant RNA.

The short term fate of the transplanted cells was studied by *in situ* hybridization using a cRNA probe specific for vector-derived sequences in the recombinant transcript. Analysis of liver sections revealed transduced hepatocytes in a periportal distribution. This is consistent with the hepatocytes seeding in sinusoids soon after leaving the portal venule.

These studies illustrate several important points regarding the feasibility of *ex vivo* gene therapy for FH. Reconstitution of only 4% of LDL receptor expression at the RNA level leads to a 30-40% decline in total serum cholesterol. This observation is consistent with studies in FH homozygotes which have demonstrated an inverse correlation between residual LDL receptor activity and serum cholesterol (41).

We next sought to modify the protocol to achieve more prolonged, if not permanent, metabolic improvement. Our strategies to improve the therapy are based on the hypothesis that the rapid deterioration of LDL receptor function in the allogeneic *ex vivo* experiments is due to rejection caused by immune responses to the allogeneic cells or to the human LDL receptor protein.

We have overcome these potential problems in a second series of experiments (18). A full-length cDNA clone for rabbit LDL receptor was isolated and cloned into retroviral vectors. High titer amphotropic producers were made with a vector that expresses rabbit LDL receptor from a β-action promoter. Viruses that express the *lacZ* gene from the viral LTR were used in control experiments. A group of WHHL rabbits underwent partial hepatectomies and the liver tissues were used to prepare hepatocytes and establish primary cultures. The cultured hepatocytes were exposed to the second generation viruses described above, harvested, and transplanted into the spleen of the animals from they were derived. The majority of the cells immediately passed through the spleen into the portal circulation and seeded the liver. Animals transplanted with LDL receptor transduced hepatocytes (N=5) demonstrated a 30-50% decrease in total serum cholesterol that persisted for the duration of the experiment (122 days). No significant change in serum cholesterol was noted in animals that received *lacZ* transduced hepatocytes (N=7). RNase protection assays demonstrated recombinant derived LDL receptor RNA in liver and spleen tissues harvested up to 6.5 months after transplantation. No diminution in recombinant RNA was noted during this time period. Albumin expressing cells were detected in spleens of transplant recipients indicating that the transplanted cells retained differentiated function *in vivo*. Transplant recipients did not develop a serologic response to wild type rabbit LDL receptor protein as determined by Western blot analysis.

These studies represent a substantial improvement over previous experiments of allogeneic hepatocyte transplantation [i.e., WHHL hepatocytes expressing human LDL receptor protein (16) or hepatocytes derived from a wild type rabbit(17, 89)] in WHHL rabbits which demonstrated transient metabolic effects in the absence of immunosuppressive therapy . The only available prolonged treatment of FH, allogeneic orthotopic liver transplantation, also requires immunosuppression with its associated morbidity (5-8). The major advantage of *ex vivo* gene therapy with autologous hepatocytes, as demonstrated in this animal model, is that long term function can be achieved in the absence of immunosuppressive therapy. Our experiments also address the potential complication of destructive immunological responses to the recombinant LDL receptor protein in recipients that had not previously been exposed to LDL receptor proteins. Functional stability of the transduced cells *in vivo* and the absence of a serological response to the wild type receptor suggests that immunological rejection may not be a confounding or limiting problem.

2. Isolation and Transduction of Human Hepatocytes

A portion of liver tissue from 4 different donors, labeled Z1 through Z4, was used for hepatocyte isolation (19). Liver sections from three of the donors were available because the organ had been surgically reduced prior to transplantation; the fourth organ had been harvested but rejected for transplantation because the donor was hemodynamically unstable and had elevated liver function tests. The organs were perfused with UW solution (Z1, Z2, Z3) or Euro-Collins solution (Z4) and stored for variable periods of time prior to hepatocyte isolation (30 min to 36 hrs). The age of the donor ranged from 22 months to 44 years.

Hepatocytes were isolated by collagenase perfusion of the major vessels using a modification of the technique shown to be successful in isolating hepatocytes from rabbits (16). The viability of recovered hepatocytes ranged from 85 to 98 %, while the recovery of viable cells ranged from 3 to 33 x 10^6 cells/gm wt wet of tissue. The hepatocytes were plated overnight in hormonally defined medium (HDM) containing fetal bovine serum onto Primaria tissue culture plates, and were subsequently maintained in serum free HDM. Plating efficiencies ranged from approximately 50 to greater than 95 %. The cultures were seeded at various subconfluent densities and rapidly grew to confluence within 72 hours of the initial plating. Hepatocytes were maintained in primary culture for a total of 4 to 5 days.

A series of experiments were performed to maximize the efficiency of retroviral mediated gene transfer. Two recombinant retroviruses were used to infect the primary cultures from each patient; both types of virus were produced in the amphotropic packaging cell line Ψ CRIP (90). The first recombinant virus was produced from the previously described BAG vector that expresses the *lacZ* gene from a transcript initiated at the 5' LTR and a gene encoding Neomycin from SV40 sequences located internal to the viral transcriptional unit (91). The second recombinant virus was produced from a vector called AFP-BA-rLDLR which expresses rabbit LDL receptor from a transcript initiated at promoter sequences that are derived from the chicken β-actin gene along with enhancer sequences from the mouse alpha-fetoprotein gene (92). The relative titer of each virus was estimated by infecting subconfluent plates of NIH3T3 cells and harvesting total cellular DNA for Southern blot analysis. BAG infected cells contain approximately 0.5 copy of proviral DNA per cell while LDL receptor infected cells contain approximately 1 copy of proviral DNA per cell.

Based on our previous experiences with primary cultures of rat (93) and rabbit (14, 18) hepatocytes, we developed a protocol for efficiently infecting human primary hepatocytes (19). The protocol was designed to maximize gene transfer and minimize the time the hepatocytes are maintained in culture. Hepatocytes were seeded at different densities (2 or 4 x 10^6 per 10 cm plate) and exposed to the viruses for a single 12 hour period beginning 48 hrs (day 2) or 72 hrs (day 3) after initial plating. Cells were harvested 48 hrs after completion of the infection and total cellular DNA was prepared for blot analysis to estimate the abundance of integrated proviral DNA in the unselected population. Efficiency of gene transfer with the LDL receptor virus ranged from a maximum of 1 proviral copy/diploid genome for Z1 to a minimum of 0.1 proviral copies/diploid genome for Z2. Infection efficiency was consistently highest when the cells were exposed to virus 48 hrs after plating; density of plating did not consistently affect the efficiency of gene transfer. The absolute level of gene transfer was less with the lower titer BAG virus although the effects of plating density and time of exposure to virus on gene transfer were similar.

Expression of the integrated LDL receptor provirus in human hepatocytes was studied by RNA blot analysis which measures the steady state level of recombinant RNAs. The AFP-BA-rLDLR vector contains two transcriptional units: transcription from the 5' LTR produces a 5.6 kb RNA responsible for passage of the virus, while a transcript initiated at the β-actin

promoter produces a 3.4 kb transcript responsible for translation of the LDL receptor protein. The 3' LTR in the plasmid vector, which has been deleted of enhancer sequences, forms the template for both LTRs in the integrated provirus (94). The rationale for this modification is to minimize the LTR transcript and maximize the translated β-actin transcript in the infected cells. Both the 5.6 kb and 3.4 kb recombinant derived LDL receptor transcripts were detected in infected hepatocyte cultures from each donor. As expected, the smaller transcript initiated from the internal promoter was more abundant than the larger LTR initiated transcript.

Hepatocyte cultures transduced with either the BAG or the LDL receptor virus were analyzed for recombinant gene expression using cytochemical analyses. Expression of E. *coli* β-galactosidase in the BAG transduced cells was detected with a histochemical stain using the chromogenic substrate X-gal that produces a diffuse blue cytoplasmic precipitate (91). This analysis demonstrated *lacZ* expression in approximately 10 to 25 % of cells thereby confirming the estimate of gene transfer provided by Southern blot analysis (0.3 proviral copies per cell). Expression of recombinant LDL receptor protein was analyzed using an *in situ* functional assay which detects uptake of fluorescent labeled LDL (14). Mock infected hepatocytes demonstrated low levels of fluorescence representing uptake via the endogenous receptors. Analysis of LDL receptor transduced hepatocytes revealed a subpopulation of highly fluorescent cells comprising approximately 20% of the culture. This result indicates that the recombinant derived receptor is functional and can be expressed at levels in far excess of the normal endogenous receptor.

3. *Ex Vivo* Gene Therapy in Nonhuman Primates

a) Aims.

i) To demonstrate the feasibility of performing *ex vivo* gene therapy at a scale that would be used in humans.

ii) Assess the short term toxicity and risks of the proposed procedures in a nonhuman primate.

b) Rationale and Experimental Strategy.

These experiments were designed to exactly simulate the proposed human experiment using nearly identical reagents and methods. The only conceptual difference between the human experiments and the baboon studies relates to the potential immunological consequences of *ex vivo* gene therapy. In the former studies, human LDL receptor is expressed in a genetically deficient human, whereas, in the latter studies a human LDL receptor is expressed in a normal baboon.

The basic design of the experiment is summarized in Table 2. The animal is subjected to a preoperative evaluation to identify any pathology that may confound the experiment. On day 0 the animal is taken to the operating room where it undergoes a partial hepatectomy (resection of the left lateral segment) and placement of an indwelling catheter into the portal circulation. The catheter is inserted into the proximal segment of the inferior mesenteric vein and the vein is ligated distally. The distal end of the catheter is tunneled subcutaneously to a posterior position and exited percutaneously between the scapulae where it is inaccessible to the animal.

The resected liver tissue is immediately taken to the laboratory where it is perfused with collagenase to release hepatocytes. The resulting cell suspension is plated in primary culture on tissue culture plastic at a density of 2 to 4 x 10^6 cells per 10 cm plate. Viral stocks from the human LDL receptor #132-10 are placed on the primary cultures for a 12-16 hr period approximately 48 hours after the initial seeding of hepatocytes. Following this exposure

to virus, the cells are harvested with trypsin and resuspended in normal saline containing heparin. In preparation for cell infusion the animal is anesthetized and taken to the fluoroscopy suite where a portal venogram is performed through the indwelling catheter to document the patency and placement of the catheter as well as the patency and integrity of the portal circulation. The cells are then infused into the catheter via the indwelling catheter in two aliquots (25 ml of cell suspension per aliquot each infused over 10 min).

In our initial experiments we plan to leave the catheter in place following cell infusion and to flush it with a heparin containing solution approximately 2-3 times per week. The purpose of this is to determine the length of time that the catheter will stay patent and in the vessel. Approximately 2 weeks after cell infusion, the animal will be taken to the fluoroscopy suite where a portal venogram will be done to assess catheter placement and patency. A laporotomy will then be performed to identify any intra-abdominal pathology and to obtain a small amount of liver tissue for histological analysis.

Table 2. Protocol For Preclinical Studies in the Baboon

Time	Event
(-) Day 7	- Clinical evaluation to include blood chemistry/hematology
Day 0	- Liver resection and catheter placement Tissue/blood saved for analysis
	- Hepatocytes isolated and plated in primary culture
Day 2	- Viral supernatant placed on cultured hepatocytes
Day 3	- Patency of catheter checked by portal venography
	- Hepatocytes isolated and infused into catheter
	- Cells and supernatants saved for analyses
Day 10	- Blood obtained for analyses
Day 13	- Patency of catheter checked by portal venography
	- Laparotomy to assess gross pathology and to obtain liver tissue for histology.

c) Results of first baboon experiment

We have completed our first baboon experiment with a second experiment planned for September or October. The first animal was a 17 kg juvenile male baboon (B206). A summary of the experimental results is provided in Table 3. The animal was taken to the operating room where the left lateral segment of the liver was resected without complication (See operative note). A Broviac catheter was placed in the inferior mesenteric vein as described above. The liver tissue, weighing 35 gm, was taken to the laboratory where it was perfused with collagenase to release hepatocytes. Approximately 0.7×10^9 cells were recovered with 98 % viability based on exclusion of trypan blue. The cells were plated onto 225 10 cm Primaria

plates and exposed to the human LDL receptor expressing retrovirus (#132-10) 48 hrs after seeding. Following 12-16 hrs of exposure to virus the cells were washed and harvested. A total of 2 x 10^9 cells were recovered with 98 % overall viability. Analysis of several plates of cells demonstrated gene transfer and high level of LDL receptor expression in 30 % of the cells. The animal was taken to the fluoroscopy suite and a portal venogram was done demonstrating patency and the correct placement of the catheter as well as a completely patent portal circulation. The hepatocytes were infused into the catheter in two aliquots as described above without apparent toxicity. Cell infusion was associated with a small increase in portal pressure which was transient (see Table 3).

Table 3. Summary of First Baboon Experiment

1. Animal - 17 kg male baboon

2. Liver resection and isolation of hepatocytes (day 0)
   - left lateral segment removed (35 gm)
   - Broviac catheter placed in inferior mesenteric vein (portal pressure - 7 cm H_2O)
   - 0.7 x 10^9 cells isolated with 98% viability
   - cells plated onto 225 10 cm plates
   - animal tolerated procedure extremely well

3. LDL receptor transduction of the cultured hepatocytes
   - DiI-LDL assay - high level overexpression of LDL receptor in 30% of the cells

4. Harvest and infusion of hepatocytes (day 3)
   - Hepatocytes harvested in two batchs:
     Batch #1 - 1.2 x 10^9 with 97 % viability
     Batch #2 - 0.8 x 10^9 with 98% viability
   - Hepatocytes infused
     Catheter and portal circulation fully patent by portal venography
     Cells Infused in two aliquots without complication
     Summary of portal pressures

initial catheter placement (day 0)	7 cm H_2O
prior to infusion #1 (day 3)	4 cm H_2O
after infusion #1 (day 3)	10 cm H_2O
after infusion #2 (day 3)	14 cm H_2O
at laporotomy (day 13)	9 cm H_2O

   - animal tolerated the procedure extremely well

5. Laporotomy (day 13)
   -Catheter found detached from mesenteric vein, no gross pathology
   -portal venogram demonstrated fully patent portal vein and intrahepatic portal venous branches with no evidence of intraluminal filling defects
   -pressure - 9 cm H_2O
   -Needle biopsy of liver performed (no histopathology noted)

In the human protocol we would have withdrawn the catheter immediately after the infusion of cells. In the baboon experiment, we elected to keep the catheter in place for a longer period of time to determine its long term stability and patency. This was complicated by the fact

that it was impossible to flush the catheter more frequently than 2-3 times a week (the animal had to be anesthetized each time) and it was impractical to completely restrain the animal from dislodging the catheter. We were able to flush and/or withdraw blood from the catheter for at least 7 days after the cell infusion suggesting that the catheter was in place and patent. By day 10, however, we were unable to withdraw blood. On day 13 the animal was taken to the operating room where a laporotomy was performed. As suspected, the catheter was detached from the inferior mesenteric vein. There was no intra-abdominal gross pathology; the sites of partial hepatectomy and catheter insertion were well healed. An intraoperative portal venogram revealed a fully patent portal vein and intrahepatic portal branches without any evidence of intraluminal filling defects. The portal pressure was essentially identical to the pressure measured when the catheter was initially placed. A needle biopsy of the liver was taken. No histopathology was noted.

The postoperative course of this animal has been unremarkable. Extensive analysis of blood chemistries and hematologies revealed no abnormalities except for a minor and transient increase in liver function tests immediately after partial hepatectomy (Table 4).

The operative note from the partial hepatectomy, tube placement and cell infusion is provided below.

Op Note on partial hepatectomy and catheter placement (8/19/91):

Anesthesia- Ketamine and atropine. Intubated without difficulty.

Prep- Shaved over abdomen and left shoulder blade. Position for Hickman marked by placing B206 into vest. Tied flat with sterile dressings underneath to allow catheter placement. Prepped with Betadine spray.

Operation- Left subcostal incision, well muscled; more difficult to go through the rectus with no muscle relaxant. Peritoneal cavity entered and adhesions to anterior abdominal wall encountered. Triangular ligamentous attachments to the left lobe were identified and divided with the cautery back to the vascular pedicle. A medium Satinsky was placed across the pedicle and clamped. The clamp was placed very carefully to avoid encroachment on the remaining hepatic veins. The liver was transected and a centimeter of tissue was left. Blood loss was minimal. An O vicryl on an SH needle was used to transfix the vascular pedicle just under the clamp. The liver remnant was trimmed carefully so as not to cut the suture. The adhesions to the anterior abdominal wall were divided and the inferior mesenteric vein was identified. Overlying peritoneum and small branches were divided. The vein was dissected for about 1.5 cm. Two 3-0 silks were used to occlude the vein. Next, the tunnel to the posterior scapular region was made by rolling B206 onto its right side and making a skin incision. The tunnel was made and the catheter was pulled through so that the Dacron cuff was about 2 cm beneath the skin. The catheter was brought through the abdominal wall lateral to the rectus and tailored to size. A partial transection of the IMV was made with about 5 ml blood loss due to getting the catheter into the vessel. It was necessary to get suction. The catheter was tied in the vein so that about 2 cm remained in the vessel. Good blood return was seen in a syringe of heparinized saline (saline containing 1-10 u/ml). The fascia was closed in two layers with O vicryl running and the skin was closed with staples. Sterile furacin spray and dressings were applied. The catheter was secured with a 3-0 nylon suture. The vest was applied and the baboon was allowed to recover in its cage.

Op Note on cell infusion (8/22/91):

Animal sedated and intubated and transported to fluoroscopy suite. Placed on fluoroscopy table and the catheter was irrigated. Good blood flash. 27cc of Conray 60 was then used to shoot two films. The first was underpenetrated. Half of the cell infusate

arrived and was infused after mixing with 2.5cc of 1000 u/ml heparin. The solution was frequently agitated and infused at a rate of 2.5 ml/min (25 ml total;10 minute infusion period). The rest of the cell infusate arrived and were infused in the same manner. B206 was under anesthesia for the same length of time as for the original operation.

Table 4. Blood Chemistries and Hematologies From the First baboon Experiment.

	Normal	Data From Time Points						
	Controls	8-13	8-20	8-22	8-23	8-26	8-29	9-5
Chemistry								
Glucose	50-129	77	75	109	105	109	65	87
Creatinine	0.8-1.4	1.3	1.5	1.4	1.3	1.09	1.44	1.64
BUN	9-25	14	16	8	8	14	10	19
Cholesterol	68-232	85	80	82	76	74	85	87
Triglycerides	21-75		69	37		49	79	65
T.Protein	5.8-7.8	6.4	6.6	6.5	6.1	6.4	7.51	7.2
Albumin	2.9-4.2	4.7	4.4	4.0	4.0	4.2	4.5	4.91
Globulin	2.4-4.4	1.75	2.1	2.6	2.2	2.2	3.0	2.29
T.Bilirubin	0.3-0.7	0.19	0.16	0.33	0.31	0.1	0.2	0.07
Calcium	8.0-9.6	9.9	9.9	9.1	9.1	9.2	10.1	10.6
Phosphorus		5.8	7.4	7.0	6.3	6.2	8.1	8.2
SGOT	16-39	62	139	47	67	53	41	31
SGPT	12-81	57	141	109	118	66	60	33
LDH	99-488	308	550	240	214	227	711	149
Alk.Phos.	154-1105	455	511	428	389	340	559	454
GGTP		28	29	27	26	24	26	28
Sodium	143-158	144	149	141	142	147	144	142
Potassium	3.2-4.3	4.2	4.1	4.0	3.8	4.0	4.0	3.5
Chloride	104-118	106	97	96	98	99	105	108
CPK		742	2877	667	975	93		473
Hematology								
HGB	8.7-13.9	14.0	13.4	13.9	13.3	12.2		13.4
HCT	31-43	42						
WBC	5.9-20.8	4.6	5.9	5.2	4.1	3.7	4.2	4.7
segs%	22-85	68	74	52	34	28	43	34
lymphs%	12-75	28	18	41	52	66	48	56
eos%	0-5	0	1	0	5	3	5	2
baso%	0-1	0	0	1	2		2	0
mono%	0-4	4	7	6	7	3	2	8
PLT	205-451	354	351				483	

III. Experimental Design

A. Overall Summary

We propose to perform *ex vivo* gene therapy on three patients with homozygous FH. A group of patients with advanced CAD who have a poor prognosis will be considered in these initial experiments. This treatment will be considered an adjunct to more traditional therapies which will be resumed 6 weeks after the gene therapy. Prognosis will be determined during a

careful pretreatment evaluation that may include cardiac catheterization. Patients will be excluded from the protocol if 1) cardiac evaluation indicates a relatively good prognosis, 2) operative risks are too great, and 3) a second dyslipidemia or concurrent hepatobiliary disease is present. Children and adults will be considered potential candidates.

The important steps in the evaluation and treatment of the patients are summarized in Table 5. The indicated time frame is based on defining Day 0 as the time of liver resection.

Table 5

Events	Time
1. Begin diet, withdraw drugs, receive last plasma exchange	(-) 6 weeks
2. Admit to CRC for 2-3 days Initial evaluation will include physical exam, history, blood work, echo with doppler, and possibly ETT or cardiac catheterization	(-) 4 weeks
3. Biweekly blood draws to establish metabolic baselines	(-) 4 weeks to (-) 1 week
4. Admit to CRC for gene therapy	(-) day 7 to (-) day 10
A. Preoperative evaluation	(-) day 7 to (-) day 1
B. Liver resection	day 0
C. Hepatocyte infusion	(+) day 3
D. Post operative evaluation	(+) day 3 to (+) day 10
5. Post treatment evaluations as outpatient	
A. Biweekly blood work x 4 weeks	(+) day 10 to (+) week 4
B. Weekly blood work x 6 months	(+) week 4 to (+) month 7
C. Resume pretreatment therapies	(+) week 6
D. Percutaneous liver biopsy	(+) month 3

The patient will undergo preliminary evaluation during a 6 week period prior to the treatment to assess their suitability for the procedure and establish metabolic baselines. The patient will then be admitted to the hospital for the gene therapy which will include the following procedures. The patient will be subjected to partial hepatectomy to remove the left lateral segment of the liver. This biopsy represents approximately 20% of the total liver by mass and weighs approximately 70 gm if removed from a 4 year old. Approximately 5×10^9 hepatocytes will be isolated from the biopsy and the cells will be plated in primary culture. Recombinant retroviruses capable of transducing the gene for human LDL receptor will be exposed to the cells for a 12-16 hour period approximately 48 hours after the initial plating. Following exposure to virus (60 hours after partial hepatectomy) the cells will be harvested, washed, and infused into the portal circulation of the patient via a percutaneous catheter inserted at the time of the partial hepatectomy.

The patient will be analyzed for engraftment of functionally corrected hepatocytes at several levels. A series of measurements will be performed to assess the effect of hepatocyte engraftment on cholesterol metabolism *in vivo*. In addition, liver tissue will be harvested 3 months after the gene therapy by percutaneous liver biopsy and will be analyzed for the

presence of recombinant derived RNA and DNA using a variety of molecular techniques. We have not included as part of the protocol an assessment of the effect of gene therapy on morbidity or mortality due to CAD. This would be extremely difficult in light of the heterogeneity of the disease and the limited number of patients to be enrolled in the study. The patients will also be carefully monitored for immunological responses to the recombinant derived receptor protein as well as morbid sequalae of cell transplantation and/or gene transfer.

We believe that this therapy has a reasonable chance of diminishing the steady state level of cholesterol in the recipient which could blunt the progression of CAD. An additional benefit from these experiments is that we will learn more about the feasibility of *ex vivo* gene therapy which will be extremely helpful in the development of other morbid inherited metabolic diseases.

B. Patient Evaluation and Selection

Homozygous FH is an extremely morbid disease which leads to premature death due to sequelae of coronary artery disease (2). The natural history of homozygous FH was reviewed in Section II.B.3. In brief, the level of residual LDL receptor activity is an important predictor of outcome: receptor negative patients (those that retain less than 2% residual receptor activity) die at an average age of about 11 to 12 years old (42, 43), whereas, receptor defective patients (those that retain greater than 2% residual receptor activity) die at an average age of 18 to 26 years old (43-48). There is no proven effective therapy for this disease, however, most patients are treated with plasma exchange or LDL-apheresis, or intensive pharmacologic therapy (2).

The patient population we are targeting for first attempts of gene therapy are homozygous FH patients whose CAD is advanced to the point where their prognosis is poor but they are still acceptable surgical candidates. We have elected not to subject this experimental therapy to patients whose prognosis is relatively good because of the relatively unknown risks associated with the procedure. Patients will be subjected to an extensive evaluation in order to assess their eligibility. A flow chart of this evaluation is provided in Figure 1.

Patients with documented homozygous FH and symptomatic CAD will be evaluated for eligibility in this protocol. The diagnosis of FH will be based on the criteria of Sprecher *et al.* (43) which include a) elevated LDL cholesterol (>500 mg/dl), b) autosomal dominant mode of inheritance, c) early onset tendinous and tuberous xanthomas, and d) LDL receptor binding in cultured fibroblasts less than 20% of normal. An assessment of LDL receptor activity in fibroblasts will also be used to stratify this population of patients into low (receptor defective) and high (receptor negative) risk groups. Symptomatic CAD will be defined by the presence of angina pectoris or a history of myocardial infarction.

Previous studies reviewed in Section III.B.3 indicate that receptor negative patients with symptomatic CAD have an extremely poor prognosis; average age of death is approximately 11 to 12 years of age (42, 43). This group will, therefore, be considered candidates for gene therapy and will be further evaluated by the pediatric or adult cardiology service to assess the risk of surgery. The specific evaluation to be performed will depend on the consult services as well as the age and condition of the patient. A general approach to this evaluation is summarized in the flow chart (Figure 1) and described below.

Initial cardiac evaluation of this group will include a history and physical exam, and an echocardiogram with doppler to assess left ventricular function and the possible existence of supravalvular or valvular aortic stenosis. Patients will be excluded from the protocol by the presence of any one of the following: 1) unstable angina pectoris, 2) left ventricular ejection fraction less than 30%, 3) decompensated congestive heart failure, 4) untreated ventricular

tachycardia, and 5) moderate to severe aortic stenosis (95, 96). Patients who are not excluded following this initial evaluation will be considered candidates for gene therapy if the operative risks are considered acceptable by the consultation services. It is likely that the patient will undergo a noninvasive evaluation for CAD preoperatively such as an exercise or dipyrimodale stress thallium test, and possibly a coronary angiogram. This will be left to the discretion of the consultation services.

We will also consider the lower risk, receptor-defective, patients if their CAD has advanced to the point where their prognosis is poor. The studies reviewed in Section II.B.3 on the natural history of homozygous FH do not specifically stratify the receptive defective population with respect to relative risk of mortality due to CAD. We will attempt to identify patients within this group who have increased risks of CAD by extrapolating from studies on normolipidemic populations.

The most important predictor of mortality due to CAD in the normolipidemic population is the anatomy of the coronary arteries as determined by coronary angiography. A selected review of this literature is provided below. Lim *et al.* followed a group of 141 patients with disease of the left main coronary artery (97). Mortality at 1 year was 22% and at 5 years was 52%. Prognosis was substantially worse in patients with decreased left ventricular function. Bruschke *et al.* determined the 5-year cardiac mortality in patients with symptomatic CAD who underwent cardiac catheterization (98). Mortality was highest in those with 3 vessel disease (54%) or left main coronary obstruction (57%). These data provide a maximal estimate of survival for FH homozygotes who have the same coronary anatomy and left ventricular function. One would expect, however, that the progression of CAD to be much more accelerated in FH homozygotes. Therefore, the prognosis may be much worse in the homozygous FH population than predicted by the studies in the normolipidemic population. This hypothesis is supported by the study of Kramer *et al.* where progression of CAD was quantified by serial coronary angiograms in several groups of patients symptomatic with CAD including FH heterozygotes, FH homozygotes, and patients with no dyslipidemias (99). CAD progression was greater in heterozygotes than in normolipidemic patients, and was 3-fold greater in homozygotes than in heterozygotes. We propose that FH homozygotes with 3 vessel or left main disease have 1 and 5 year survival rates significantly worse than 20 and 50%, respectively.

A second group of receptor defective patients that likely has a relatively poor prognosis are those who have undergone coronary revascularization (usually for triple vessel or left main disease). An estimate of survival in this group again must be extrapolated from a broader experience in the normolipemic population. Patients undergoing coronary revascularization have a slightly improved survival over those treated medically (100). The prognosis in FH homozygotes will likely be much worst because even modest hypercholesterolemia accelerates the rate of reocclusion of the bypass grafts (101).

The strategy for evaluating receptor defective FH patients is summarized in Fig. 1. Receptors defective FH homozygotes that have symptomatic CAD will undergo a noninvasive cardiac evaluation to identify any contraindications which would exclude the patient from the protocol. The exclusion criteria have been listed above. Patients with no contraindications will undergo further testing to better assess prognosis. Any patient with a history of coronary revascularization will be considered to have advanced disease and therefore will be a candidate. A coronary angiogram will be performed in the remaining patients to identify those with severe disease that have a relatively poor prognosis. Severe disease will be defined as 1) significant stenosis of the left main coronary artery, or 2) stenosis and/or occlusions in all three of the major coronary arteries (right coronary artery, left anterior descending coronary artery, circumflex artery). Patients with mild to moderate CAD have a more favorable prognosis and would not be eligible for gene therapy because the risks of this invasive and innovative therapy would no justify the benefits in this relatively stable population. Patients with severe disease

will be evaluated for coronary revascularization or angioplasty prior to and irrespective of the gene therapy. These patients will be considered candidates for gene therapy after this evaluation is completed and the recommendations are implemented (i.e., coronary revascularization, angioplasty, or medical therapy).

Patients will also be evaluated for evidence of a second dyslipidemia or hepatobiliary disease. Evidence to suggest the presence of either concurrent condition will exclude the patient from participating in this study.

An initial evaluation will be done 1 month prior to the procedure. The purpose of this evaluation is to establish the eligibility of the patient for the protocol and to establish baseline metabolic parameters. The patient will be placed on an American Heart Association Phase III diet 2 weeks prior to the initial evaluation. All drugs that may affect cholesterol metabolism and plasma exchange or LDL apheresis treatments will be discontinued two weeks prior to the visit. The following studies will be done:

i. History and physical exam

ii. General laboratory studies; and rule out hypothyroidism, azotemia, significant proteinuria, diabetes, obstructive hepatobiliary disease:

CBC, platelets, differential
Erythrocyte sedimentation rate (ESR)
Complete urinalysis
Test panel II; electrolytes, glucose, creatinine
Test panel IV; calcium, phosphorus, protein, albumin, bilirubins, ALT, AST, alk phos, LDH, uric acid,
Glycosylated hemoglobin
TSH

iii. Assess liver function;
above, plus protein electrophoresis

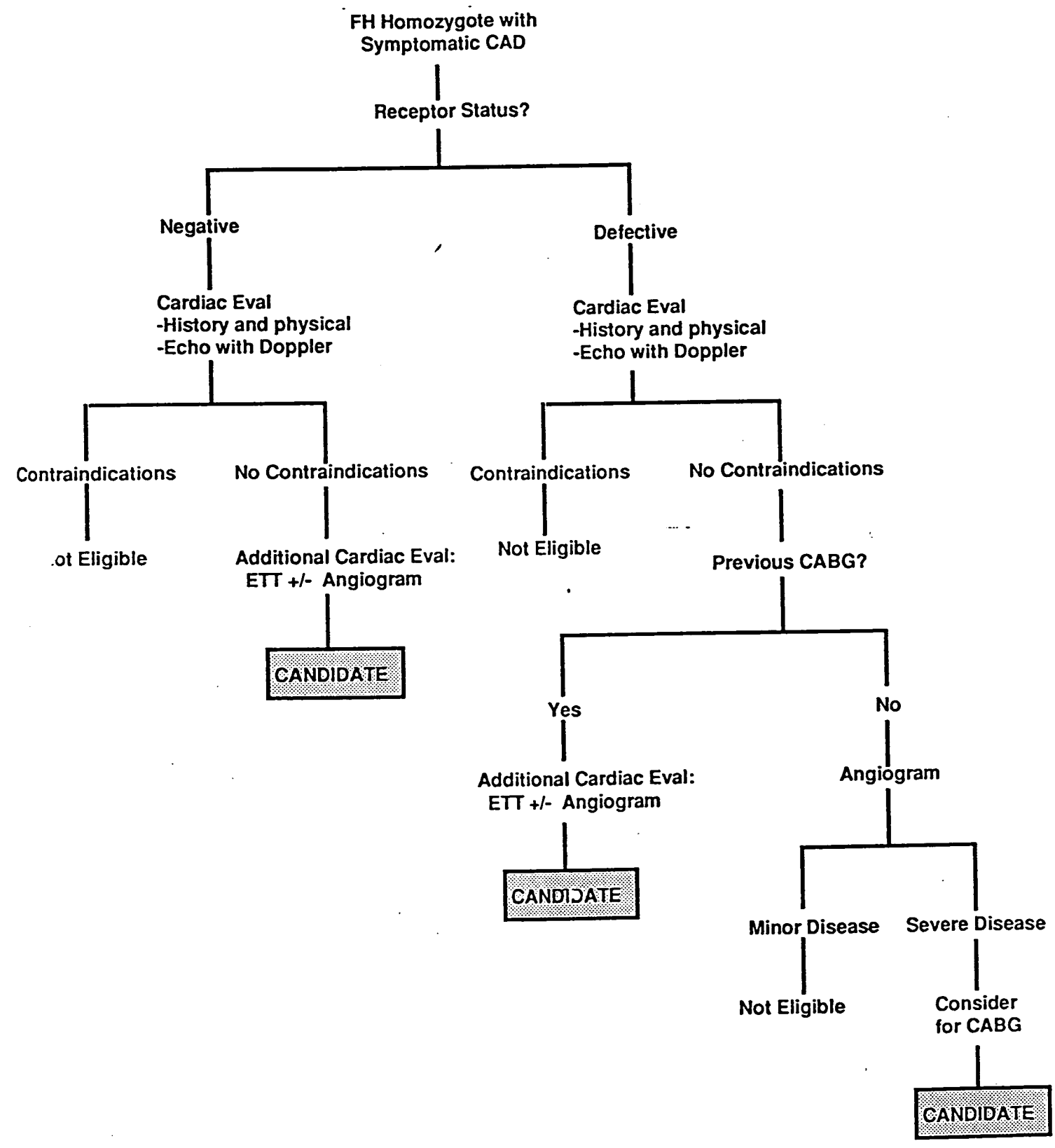
iv. Assess coagulation status:
Prothrombin time
Partial thromboplastin time
Bleeding time

v. Assess lipid/lipoprotein status:
Fasting cholesterol, triglycerides, HDL-C, LDL-C
Lipoprotein electrophoresis
LPP fractionation
CHOL & TG in LDL, IDL, VLDL, HDL, and chylos
Apo AI, AIV, B100, CII, CIII, E, AI, A2
Free fatty acids

vi. Assess other atherogenic potentials:
Fibrinogen
PAI
LP (a)

vii. Cardiac evaluation:
EKG
Echo with Doppler

PATIENT EVALUATION TO DETERMINE ELIGIBILITY FOR GENE THERAPY



C. Vectors and Viruses

1. Vectors for transfer and expression of human LDL receptor

The proviral components of these vectors are mainly derived from the previously described vector DO1 (104) are presented as follows: The parent vector BA-(h)LDLR has been described in a previous publication (14). The general structure of the vector will be described below. The backbone structure of this vector includes an intact 5' LTR of Moloney murine leukemia virus (Mo-MLV) with additional Mo-MLV sequences between the 5'LTR and the internal promoter spanning nucleotide 146 at the border of U5 to the natural Xho I site in the gag coding region at nucleotide 1560 (with the exception that a Sac II linker was inserted at the Hae III site at nucleotide 624; see Ref. 105) The plasmid also contains wild-type Mo-MLV sequences from the Cla I site at nucleotide 7674 (which was converted to Bam HI site with synthetic linkers) to the end of the 3' LTR. Sequences containing the viral enhancer elements of the 3' LTR from the Pvu II site at nucleotide 7933 to the Xba I site at nucleotide 8111 have been deleted. In addition to these sequences, there are flanking mouse genomic DNA and pBR322 sequences (spanning the Hind III site to the Eco RI site). The initial promoter used in this vector was derived from a Xho I to Mbo I fragment of the chicken β-actin gene spanning nucleotides -266 to +1 (106). The Mbo I site was converted to a Bam HI site and the modified β-actin fragment was cloned into the parent vector. The LDL receptor coding sequences were derived from a 2.6 kb Hind III fragment of a full-length cDNA fragment (Ref. 54; provided by D. Russell, J. Goldstein, and M. Brown). The Hind III sites were converted to Bcl I sites and the cDNA was cloned into the Bam HI site of the vector. This cDNA fragment contains the entire coding sequence with 13 base pairs (bp) of 5' untranslated sequence (AGCTTAATACACA) and 5 bp of 3' untranslated (ATCAG). An enhancer was introduced into the Xho I site of BA-(h)LDLR vector. These sequences were derived from an area 5' to the immediate early (IE) gene of human cytomegalovirus [from Spe I (at -580 of IE gene) to Pst I (site in vector sequence) of CDM8, Ref. 107] were subcloned into PUC19. A portion containing IE enhancer sequences was removed on a Xho I (from polylinker) to Nco I (-220 of the IE gene) fragment (for numbering of IE enhancer see Ref. 108). Synthetic linkers were used to convert the Nco I site to a Xho I site and the modified fragment was cloned into the unique Xho I site of BA-(h)LDLR located 5' to the β-actin promoter. This new vector is called CMV-BA-(h)LDLR. The structure of this vector is presented in Fig.2.

2. Description of the CMV-BA (h) LDLR retroviral sequences

The complete nucleotide sequence of the proviral component of this plasmid is currently being determined by Lark Sequencing Technologies Inc. Sequence determination is being performed in compliance with FDA/EPA Good Laboratory Practices. The region to be sequenced has been subcloned as two overlapping restriction fragments into pBluescript II (Stratagene) and pGem5Zf (Promega). Nested deletion clones are being generated in both directions for each of the subclones using a modified exo III/S1 nuclease procedure. These deletion clones will be size selected to provide complete coverage of each strand and sequenced using the dideoxynucleotide termination procedure. Internal sequencing primers will be synthesized and used to close gaps between contigs and to fill in any single-stranded regions. Anticipated completion of this project is November 1,1991.

3. Isolation of the LDL receptor virus producing cell line #132-10

Viral-producing cell lines were isolated for each vector using the amphotropic packaging cell line Ψ-Crip (90). Ψ-Crip is an amphotropic packaging cell line constructed by Dr. Olivier Danos in Dr. Richard Mulligan's laboratory. A brief review of the construction of this cell line is provided below. Ψ-Crip cell line was constructed by transfecting the *gag-pol*

and *env* functions on separate constructs into NIH3T3 cells. The original paper describing the isolation of the cell line is provided in Appendix I. The 3' LTRs of the constructs were replaced with heterologous polyadenylation sequences. These modifications were performed to minimize the chance that recombination could result in the production of replication competent virus. The plasmids used to make the packaging cell line are described in the original paper.

The vector was cotransfected into Ψ-CRIP with pSV2Neo, and stably transfected clones were selected in G418 (1 mg/ml). Individual clones (25 from each transfection) were isolated and analyzed for production of virus. The producer is maintained in Dulbecco's modified medium supplemented with 10% bovine calf serum. Supernatants from confluent plates of clones were harvested and exposed to subconfluent plates of a human fibroblast line deficient in LDL receptor in the presence of polybrene (8 μg/ml). Expression of wild-type LDL receptor was assayed *in situ* by incubating the cultures with fluorescent labeled LDL (14). Fluorescent microscopy revealed substantial activity in a subpopulation of fibroblasts from each infected culture.

Freshly isolated viral supernatants were analyzed for replication competent virus using the previously described *LacZ* mobilization assay (90). NIH 3T3 cells harboring a single copy of a recombinant retroviral genome encoding E. *coli* β-galactosidase were exposed to the viral supernatant and maintained in culture for 7-10 days. A supernatant was harvested and used to infect NIH 3T3 cells which were subsequently analyzed for *LacZ* expressing cells using X-gal chromogenic assay. None of the virus producers have scored positive for replication competent virus or packaging of the psi genome using this sensitive assay.

The structure of the retroviral vector used to make this virus producer is illustrated in Figure 2. The enhancer from CMV has been cloned in reverse orientation immediately upstream to the β-actin promoter. In general, the CMV containing vectors produced much higher titers of virus than the vectors which did not contain additional enhancers or those that contained the alpha-fetoprotein enhancer.

The relative titers of several candidate producers were characterized by exposing a subconfluent plate of NIH3T3 cells to freshly isolated viral supernatants and analyzing the DNA from the infected, unselected population of cells for the presence and abundance of proviral sequences. A representative Southern analysis from this experiment is presented in Figure 3. Several infected populations demonstrated unrearranged proviral sequences at frequencies greater than or equal to 1 copy of provirus per cell. The vector does not contain a selectable marker so it is impossible to perform a limiting dilution assay. Previous experience in our laboratory suggests that a viral stock which transfers 2 proviral copies per NIH3T3 cell would be equivalent to approximately 1 to 5 x 10^6 cfu/ml in a limiting dilution assay. RNA blot analysis of total cellular RNA from the infected unselected NIH3T3 cells is presented in Figure 4. In each case, the transcript initiated from the β-actin promoter is more abundant than the LTR initiated transcript.

Viral stocks were also used to infect a diploid fibroblast cell line derived from a patient with FH. The infected unselected population of cells was analyzed for production of functional LDL receptor protein using the previously described ^{125}I-LDL binding and degradation. Many of the transduced populations of FH cells expressed levels of LDL receptor that exceeded those measured in fibroblast cells derived from a normal individual as seen in Figure 5. Finally, as described in the initial proposal none of the viral producers passage the Ψ^- genome or produce replication competent virus.

Based on the Southern and Northern experiments, as well as the ^{125}I-LDL functional assay we have selected producer #132-10 as the one to be used in the human experiments.

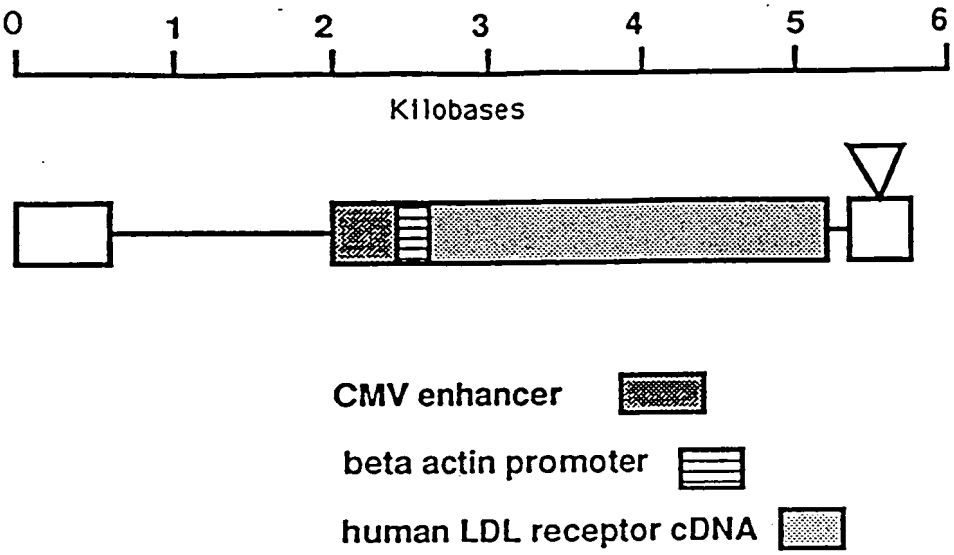


Figure 2. Proposed vector for use in gene therapy of familial hypercholesterolemia

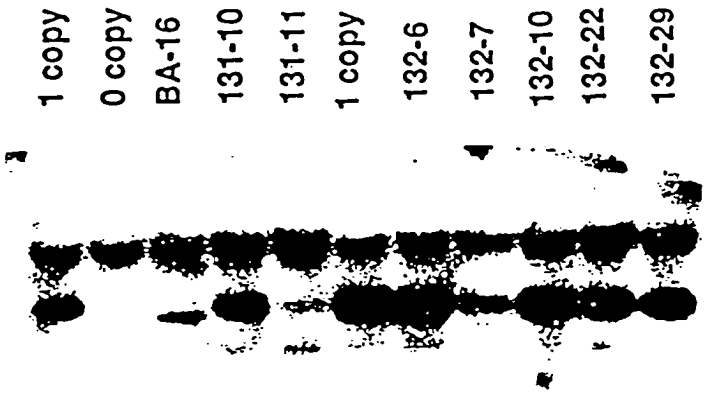


Figure 3. Southern blot analysis of NIH3T3 cells infected with the human LDL receptor retroviruses. Subconfluent plates of NIH3T3 cells were exposed to freshly prepared virus and total cellular DNA was isolated when the plates were confluent. DNA (10 μg) was restricted with Kpn I, fractionated on an agarose gel, and transferred to a filter which was hybridized with a human LDL receptor cDNA probe. 1 copy represents the addition of plasmid DNA to uninfected total cellular DNA at a level equal to a single copy per cell. The rest of the samples are DNAs from NIH3T3 cells infected with the indicated virus producers: BA-16 β-actin promoter no enhancer, 131 series β-actin promoter with the CMV enhancer in direct orientation, and the 132 series β-actin promoter with the CMV enhancer in reverse orientation.

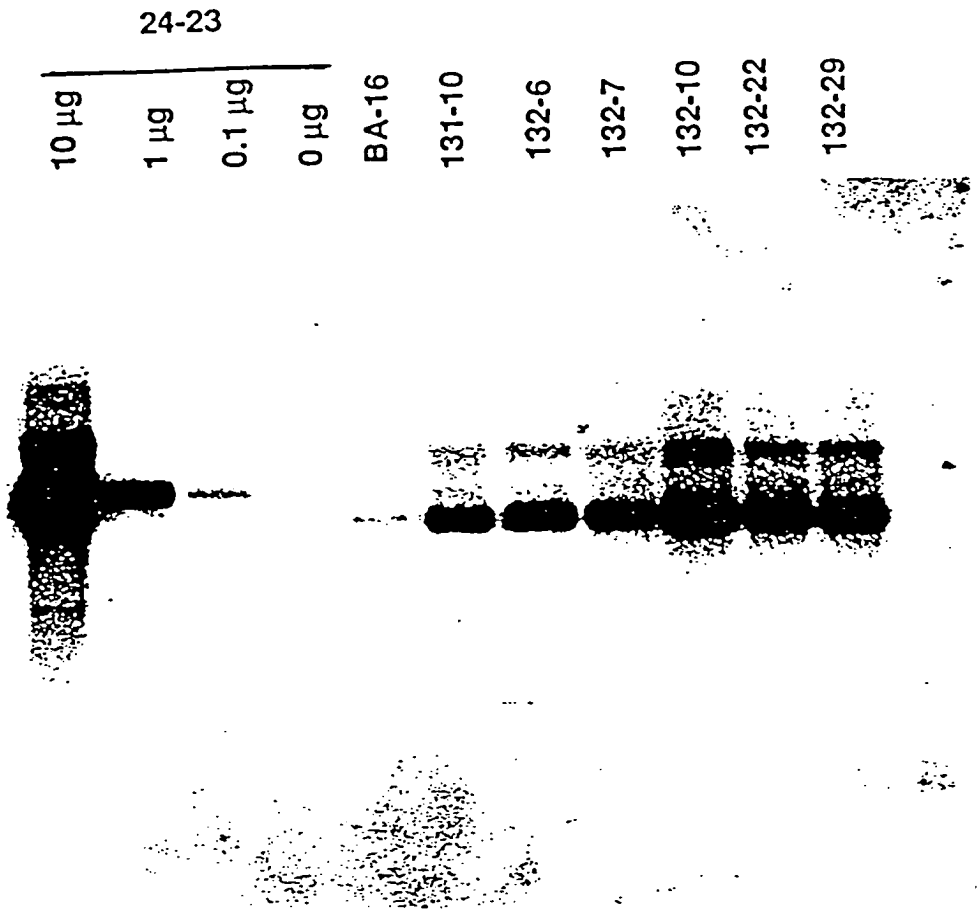


Figure 4. Northern blot analysis of NIH3T3 cells infected with the human LDL receptor retroviruses. Subconfluent plates of NIH3T3 cells were exposed to freshly prepared virus and total cellular RNA was isolated when the plates were confluent. RNA (10 µg unless otherwise noted) was fractionated on an agarose gel, and transferred to a filter which was hybridized with a human LDL receptor cDNA probe. 24-23 is RNA from a virus producer transfected with an LTR driven LDL receptor vector. The rest of the samples are RNAs from NIH3T3 cells infected with the indicated virus producers: BA-16 is β-actin promoter no enhancer, 131-10 is β-actin promoter with the CMV enhancer in direct orientation, and the 132 series are vectors with the β-actin promoter the CMV enhancer in reverse orientation.

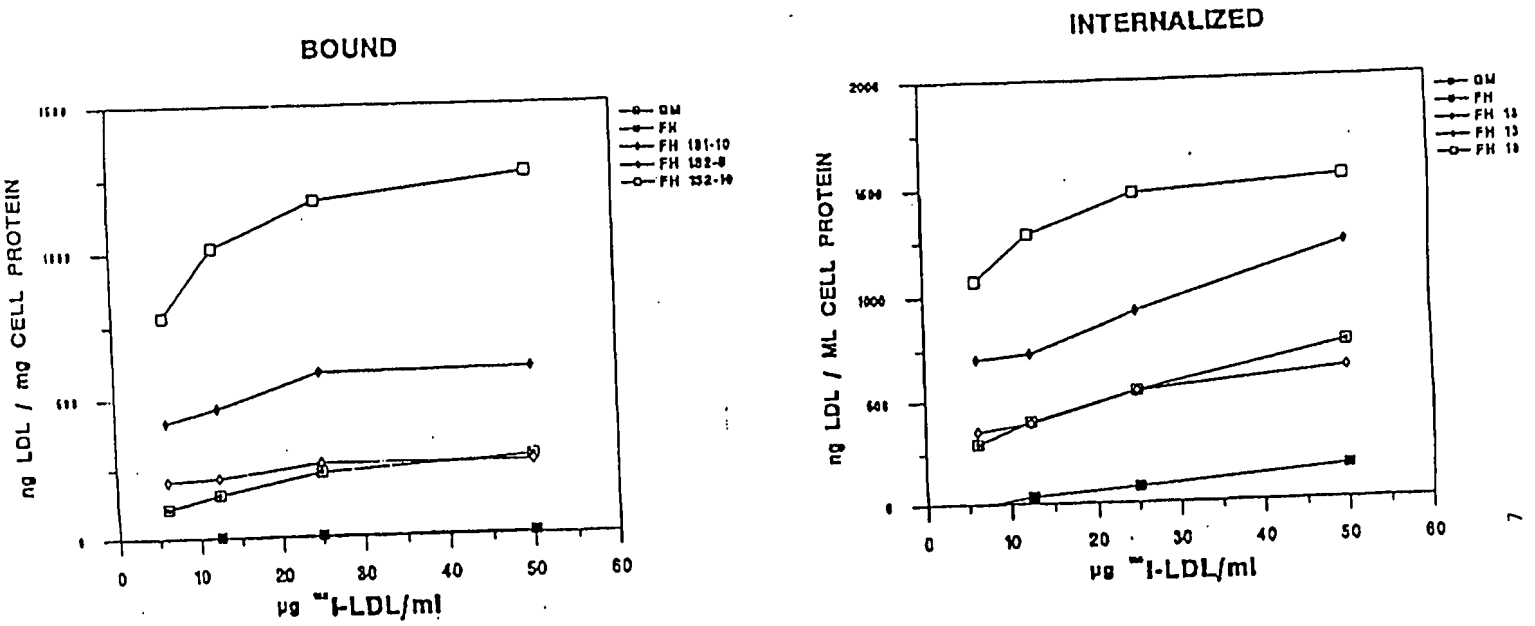


Figure 5. The measurement of surface binding and internalization of LDL were determined as described by Goldstein *et al.* (Methods Enzymol. 98: 241, 1983). LDL was prepared by sequential flotation of plasma from normal human volunteers according to the method of Havel *et al.* (J. Clin. Invest. 39: 1560, 1960), and iodinated with ICl. For these experiments LDL was labeled to a specific activity of 422 cpm/ng. 97% of the counts were precipitable by TCA. Diploid fibroblasts were grown to late log phase in tissue culture 24 well cluster dishes. LDL receptor activity was up-regulated by growing the cells for 48 hrs in media containing 10% lipoprotein-deficient fetal bovine serum (LDS). On the day of the experiment, the culture media was removed from each well. The cells were returned to the incubator for 5 hrs. The estimation of values for nonspecific binding and internalization were achieved by incubating cells with a 20-fold excess of unlabeled LDL. Values obtained are expressed as ng ^{125}I-LDL bound and internalized per mg cell protein. GM is a diploid fibroblast cell line derived from a normal patient. FH is a dipoid fibroblast cell line that was mock infected or infected with the various viruses 131-10, 132-6, and 132-10. A description of the viruses can be found in the text of the letter.

We have extensive experience with ΨCRIP as a packaging cell line in combination with the type of vector used to make CMV-BA-(h)LDLR (i.e., internal promoter, SD+, gag+, and with enhancer deletions in the 3' LTR- see Fig. 2 for basic structure). Our experience indicates that this combination is effective in producing high titer virus as well as safe in terms of production of replication-competent virus or packaging of the Ψ- genome. Between July 14,1987 and September 7,1991, we have performed 159 transfections of retroviral vectors into the ΨCRIP (94 transfections) and ΨCRE (65 transfections) packaging cell lines. Retroviral vectors of the type used to construct CMV-BA-(h)LDLR have been used in 110 of these experiments. Approximately 25 stable viral producers from each transfection have been isolated and characterized with respect to viral titer resulting in a cumulated experience of approximately 3975 individual stable producers. The highest titer stable producer from each transfection are usually screened for replication-competent virus using the *lacZ* mobilization assay. We have never detected replication-competent virus form these cell lines.

4. Characterization of the LDL receptor virus producing cell line #132-10

A master cell bank (MCB) of the #132-10 cell line, comprising of 100 ampules, has been established. We are in the process of characterizing this cell line using criteria suggested by the FDA in published documents. The following analyses of the MCB are underway. We expect these studies to be completed by November 1,1991. A brief protocol for each assay can be found in Appendix K.

Test	Performed By
Sterility	OL[1]
Cell Culture ID	OL
Mycoplasma	OL
Induction RT	OL
Extended XC Plaque	OL
Extended S+L- Focus	OL
MAP	OL
In Vivo Virus	OL
In Vitro Virus	OL
Co-cultivation with Mink Lung	OL
Co-cultivation with RD	OL
Bovine Viruses	OL
Porcine Parvovirus	OL
LDL Receptor Transduction	H.A.L.[2]

1 Outside Laboratory

2 Human Applications Laboratory, University of Michigan

We have extensively analyzed the cell line used to lay down the MCB (#132-10) with respect to 1) LDL receptor transduction and 2) production of replication-competent retroviruses with ecotropic or amphotropic host range. The producer has been analyzed for LDL receptor transduction and helper virus on multiple occasions after it had been maintained in culture for various times up to 4 weeks

Viral titer is assessed one of two ways. One approach is to harvest virus and infect a subconfluent plate of NIH3T3 cells for 12-16 hrs in the presence of polybrene (8 ug/ml). When the cells are confluent, total cellular DNA is harvested and analyzed for integrity and

abundance of proviral sequences by Southern blot analyses (see Fig.3 for example). This has been repeated two times with essentially identical results. The copy number of intact provirus was 1 copy/cell on both occasions and rearranged proviral sequences were never detected.

An alternative and complementary approach is to assess efficiency of gene transfer based on transgene expression. A viral stock is exposed to a subconfluent plate of diploid fibroblasts from a patient with FH for 12-16 hrs in the presence of polybrene (8 ug/ml). When confluent, the cells are incubated with fluorescent labeled LDL for 4 hrs and visualized by fluorescence microscopy. The relative number of fluorescent cells provides a minimal estimate of gene transfer. This analysis has been performed four different times with virtually identical results. The relative number of fluorescent cells varied from 50-80% in these experiments. The #132-10 viral producer was analyzed on three separate occasions for the production of replication-competent virus of ecotropic or amphotropic host range using the previously described *lacZ* mobilization assay. A brief description of the method is provided below. The viral supernatant is exposed to a subconfluent plate of the cell line, 3T3BAG, for 12-16 hrs in the presence of polybrene (8 ug/ml). The 3T3BAG cell line is a clone of NIH3T3 cells which harbors a single copy of a *lacZ* containing provirus. The infected 3T3BAG population is propagated in culture through two passages over a ten day period. A supernatant is then harvested from the 3T3BAG population and exposed to a subconfluent plate of NIH3T3 cells for 12-16 hrs in the presence of polybrene (8 ug/ml). When confluent, this infected population of cells is analyzed for the presence of the *lacZ* provirus using the chromogenic cytochemical assay. The packaging cell line Ψ2 which packages the Ψ- genome at a low level is always used as a positive control. Supernatant from #132-10 harvested three different times following initial seeding (up to one month) was analyzed using the *lacZ* mobilization assay (Experiment #1- duplicates, Experiment #2- duplicates, and Experiment #3- quadruplicates). Each experiment scored negative.

5. Production of the LDL receptor viral supernatant

Day 0

1. Make complete media by filtering the following components through a 0.45μm one liter filter: 1000 ml of high glucose DMEM and 100 ml of bovine calf serum

2. Use a 10 ml pipette to remove 10 ml of media from each liter to test for sterility.

3. All media will be stored at 4 ^{o}C.

4. To thaw the seed lot of #132-10, take five cryovials of each from the -135 ^{o}C and put in a 37 ^{o}C water bath.

5. Use a 10 ml pipette and add 10 ml of complete medium to 5 x 10 cm tissue culture plates.

6. Use a 1 ml pipette to transfer the cells from each cryovial to a plate.

7. Place the plate at 37 ^{o}C, 5% CO_2.

8. Two hours later, aspirate off media with a pasteur pipette and add fresh complete medium with a 10 ml pipette and return plate to 37 ^{o}C, 5% CO_2.

Day 1 (or when confluent)

1. Aspirate off media from cells with a pasteur pipette.

2. Use a 5 ml pipette to add 5 ml PBS to each plate.

3. Aspirate off PBS with a pasteur pipette.

4. Add 1 ml of Trypsin-EDTA with a 1 ml pipette to each plate.

5. Let plate sit in hood until cells just start to release from the plate.

6. Add 5 ml of complete medium to each plate with a 5 ml pipette (to stop the trypsin reaction) and transfer to a 15 ml conical test tube.

7. Spin down each test tube in a clinical centrifuge at setting 3 for 2 minutes.

8. Aspirate off the supernatant with a pasteur pipette.

9. Resuspend each cell pellet with 10 ml of complete medium using a 10 ml pipette.

10. Add 1 ml of cell suspension to a 10 cm plate (that had already been filled with 9 ml of complete medium while the cells were being spun down). This constitutes a 1:10 split.

11. Place each plate (50 total) at 37 ^{o}C, 5% CO_2.

Day 5 (or when confluent)

1. Aspirate off media from cells with a pasteur pipette.

2. Use a 5 ml pipette to add 5 ml PBS to each plate.

3. Aspirate off PBS with a pasteur pipette.

4. Add 1 ml of Trypsin-EDTA with a 1 ml pipette to each plate.

5. Let plate sit in hood until cells just start to release from the plate.

6. Add 5 ml of complete medium to each plate with a 5 ml pipette (to stop the trypsin reaction) and transfer to a 15 ml conical test tube.

7. Spin down each test tube in a clinical centrifuge at setting 3 for 2 minutes.

8. Aspirate off the supernatant with a pasteur pipette.

9. Resuspend each cell pellet with 10 ml of complete medium using a 10 ml pipette.

10. Add 1 ml of cell suspension to a 10 cm plate (that had already been filled with 9 ml of complete medium while the cells were being spun down). This constitutes a 1:10 split.

11. Place each plate (500 total) at 37 °C, 5% CO_2.

Day 8 (or when plates are subconfluent)

1. Aspirate off media using a pasteur pipette.

2. Add 10 ml of fresh complete medium to each plate using a 10 ml pipette.

3. Place each plate at 37 °C, 5% CO_2

Day 9

1. Take off supernatant using a 10 ml pipette and filter through a 0.45µm one liter filter unit. Pool 100 plates into each filter unit.

2. Repeat the above step for the other 400 plates.

3. Pool all the supernatants by pouring the supernatants together until thoroughly mixed.

4. From the 5 one liter filter bottles, remove 50 ml using a 25 ml pipette and transfer to a 50 ml conical test tube. (See section F for assays to be performed on the supernatants.)

5. Aliquot the 5000 ml of supernatant into 200 ml storage bottles.

6. Store the 200 ml aliquots at -70 °C.

6. Characterization of the LDL receptor viral supernatant

Aliquots of the pooled supernatants will be analyzed for the following tests and certified by the FDA prior to use in the human experiments:

Test	Performed By
1. Identity	H.A.L.[1]
A. Fluorescent LDL assay	
B. Southern blot analysis	
2. Sterility	O L[2]
4. General Safety	OL
5. Extended S+L- Focus	OL

1 Human Applications Laboratory, University of Michigan
2 Outside Laboratory

D. Harvest of Liver

Autologous hepatocytes will be harvested from liver tissue obtained via a partial hepatectomy of the recipient. Through a bilateral subcostal incision, attachments to the left

lateral segment of the liver will be divided. Ligamentous attachments to the left lateral segment of the liver will be taken down and mattress-type sutures will be placed in the parenchyma to occlude the vascular supply. The liver parenchyma will be transected along the line of demarcation of blood supply with a scalpel. Individual blood vessels and bile ducts will be suture ligated. The specimen will be immediately transferred to the Human Applications Laboratory for hepatocyte isolation. To alleviate the need for a second laparotomy, the left gastric (coronary) vein will be identified and isolated. A Broviac catheter will be inserted and secured into the coronary vein. The catheter will be tunnelled through the abdominal wall and secured to the skin with monofilament absorbable suture at a site distant to the laparotomy incision. After hemostasis is achieved, the abdominal wall will be closed in two layers with absorbable sutures.

E. Isolation of Hepatocytes

1. Hepatocyte Isolation and Primary Cultures.

The resected liver will be immediately transported on ice to the Human Applications Laboratory. Major vessels will be cannulated with 16 gauge angiocatheters and perfused with 1X Leffert's solution (102), pH 7.4, containing 0.5 mM EGTA for 10 minutes. This will be followed by perfusion with 1X Leffert's solution, pH 7.4, without EGTA for 5 minutes, and then 1X Leffert's, pH 7.4, 5 mM $CaCl_2$, 5 mg/ml BSA (fraction V), 0.5 mg/ml collagenase D (Boehringer Mannheim) for 18 to 24 minutes. The collagenase perfusion will be terminated when the liver softens and begins to dissociate. All solutions will be filter sterilized prior to use and oxygenated at 37^{o} during the perfusions. Flow rates will vary from 100 to 125 ml/min. The perfused liver will be teased apart with a rubber policeman and forceps, and filtered through a presterilized 85 mm nylon mesh at 4^{o} into a sterile flask containing RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (Medium A). The filtered hepatocytes will be pelleted by centrifugation three times at 50 x g for 1 min, and resuspended in medium A, and viable cells will be quantified by exclusion of trypan blue.

Hepatocytes will be plated at 7×10^5 cells per cm^2 overnight in hormonally defined medium (HDM, Ref. 103) containing 10% fetal calf serum and 1% penicillin/streptomycin. The following day the media will be replaced with fresh HDM without serum. The preparation of hepatocyte cultures will be performed in a laminar flow hood. The specific components of HDM are listed under Appendix J.

There are three possible supports onto which the cells can be plated. The approach we have the most experience with involves plating 4×10^6 cells onto 10 cm Primaria plates (16, 17, 19, 93). The expected isolation of hepatocytes from the resected liver is 2×10^9 cells. This will require 500 10 cm plates, a number we have easily managed in our WHHL and baboon experiments. An alternative approach we are actively exploring is to grow the cells in roller bottles or bioreactors.

All procedures involving the isolation, cultivation and transduction of the hepatocytes will be performed in a dedicated laboratory located in the Clinical Research Center at the University of Michigan. This facility, called the Human Applications Laboratory, is currently being renovated to become a GLP/BL2+ facility.

2. Retroviral transduction procedure of primary FH hepatocytes

FDA certified, frozen supernatant will be thawed in preparation for hepatocyte transduction. The thawed virus will be supplemented with polybrene (8 ug/ml) and placed on the primary cultures of hepatocytes. Virus will be exposed to the cultured hepatocytes for a

12-16 hour period beginning 48 hours after initial plating. The virus will be removed, pooled, and aliquots will be subjected to analyses described in section III.E.3. The hepatocyte cultures will be washed extensively with PBS, and released with Trypsin-EDTA as described (16). The detached cells will be recovered by centrifugation, pooled and washed extensively in isotonic saline. In preparation for transplantation, the cells will be suspended in isotonic saline.

3. Analysis of transduced hepatocytes

Approximately four hours prior to cell harvest, an extra plate of infected hepatocytes will be assayed for recombinant gene expression using the cytochemical assay which measures uptake of fluorescent labeled LDL. Fluorescent LDL is added to the cells for 4 hours and removed immediately prior to the cell harvest. The monolayer is visualized by fluorescence microscopy to identify transduced cells. This provides a minimal estimate of gene transfer.

An aliquot of the pooled supernatant removed from the hepatocyte cultures will be analyzed for gross contamination by fungus or bacteria using the following procedures. A 200 ml aliquot of this supernatant will be concentrated 100x by centrifugation. The sediment will be gram-stained and visualized under oil immersion microscopy. Absence of detectable gene transfer using the *in situ* LDL receptor assay, recovery of less than 5 x 107 hepatocytes or gross contamination in the gram-stained sediment will preclude reinfusion of the hepatocytes.

A more extensive analysis will be performed on the pooled hepatocyte suspension used for reinfusion. The following tests will be performed but the results will not be available until after the cells have been infused.

Test	Performed By
Sterility	[1]
Electron Microscopy	[1]
Extended S+L- Focus	[1]
LDLR gene Transduction	H.A.L.

1 Outside Laboratory
2 Human Applications Laboratory, University of Michigan

Aliquots of the pooled supernatants removed from the hepatocyte cultures immediately prior to harvest will also be analyzed for sterility, electron microscopy, and extended S+L- focus by the above Outside Laboratory.

F. Delivery of Hepatocytes

An important issue relates to the number of cells to be infused. Based on the hepatic perfusions done to date, it is possible to harvest 1 to 10 x 10^9 cells from a piece of liver that one can expect to obtain by removing the left lateral segment of a liver from a 4 yr old (approximately 70 gm). Our experience with the WHHL rabbit indicated that 2 x 10^8 cells could be safely transplanted into 2 kg animals. In the baboon experiment, 2 x 10^9 cells were safely infused into a 17 kg animal. It is reasonable, therefore, to use 1 x 10^8 cells/kg in human studies, up to the maximum number harvested. Considerations include the absolute volume to be infused, which is usually limited to 25-50 ml. It is also possible that increases in the level of LDL receptor expression achieved by transduction would allow a corresponding decrease in the number of reinfused cells that are necessary to achieve a therapeutic effect.

A wide variety of locations have been used for the implantation of hepatocytes in various animal models. To date, however, no human studies have been performed. We propose the direct re-infusion of autologous human hepatocytes into a tributary of the portal venous system. There are benefits as well as drawbacks to this approach. The ability to leave an indwelling Broviac-type catheter at the time of hepatocyte harvest allows the administration of hepatocytes without the need for further surgery or anesthesia. Patients with FH have premature atherosclerotic cardiovascular disease predisposing them to increased cardiovascular morbidity. The ability to reinfuse cells without the extra stress brought about by a second laparotomy for reinfusion is therefore useful. The patients can be studied radiologically immediately prior to hepatocyte reinfusion to insure that the vessel is open and that hepatoportal flow is present.

There are also theoretical disadvantages to the direct infusion of hepatocytes into the portal venous system. The most devastating complication of this approach would be portal vein thrombosis. Portal vein thrombosis might complicate a subsequent attempt at orthotopic liver transplantation and could lead to variceal hemorrhage and ascites. In a review of the incidence of portal vein thrombosis after the intraportal infusion of pancreatic islets, 3 of 57 patients developed portal vein thrombosis (109). The cells infused in most cases are portions of pancreatic tissue acutely digested and reinfused. The incidence of portal vein thrombosis following infusion of autologous hepatocytes should be lower because the cells are autologous rather than allogeneic, and the infusate contains a suspension of disaggregated cells rather than tissue fragments.

A number of other forms of hepatocyte delivery have been considered and rejected for a variety of reasons. The most direct methods would be the infusion of cells by interventional radiologists. There are three possible approaches: 1) hepatic arterial reinfusion via femoral puncture (110); 2) percutaneous transhepatic/transplenic injection into the portal vein (111); and 3) retrograde injection into the portal vein by a transjugular hepatic venous puncture (112). There are risks shared by all three. Bleeding, either overt or occult may be a result of these procedures. Also, the risk of portal venous, hepatic arterial or other major vascular thrombosis may occur, complicating the future performance of a hepatic transplant. Lastly, these procedures require several hours of sedation and in children would almost certainly require the use of a general anesthetic. The additional physiologic burden of a second anesthetic in a patient population at risk for adverse cardiovascular events cannot be overestimated.

There are several points to consider in the use of interventional techniques which have led us not to abandon the concept entirely. This patient population does not have a coagulopathy such as is seen in patients with fulminant hepatic failure, so that percutaneous puncture is relatively safe. In fact, this will be our preferred route of delivery in case of catheter thrombosis prior to reinfusion. Also, the cells can be directed into a subsegmental branch of the portal vein and cell migration can be further minimized by the placement of coils or gelfoam. The main theoretical advantage of this technique is the possibility of decreasing main portal vein thrombosis to a bare minimum by avoiding diffuse showering of cells throughout the portal distribution.

Laparoscopic delivery of hepatocytes into the patient is an attractive but currently unfeasible approach. Laparoscopy would also require a second anesthetic, and there are no standardized techniques for the isolation of a reasonable branch of the portal venous circulation. Such approaches will no doubt be worked out in the near future.

There are potential problems with this proposed method of hepatocyte introduction such as occlusion of the catheter prior to cell infusion. The vessels available for use in children are relatively small and are not easily kept patent once cannulated. To minimize the risks of thrombosis, the Broviac catheter will be flushed every eight hours with a heparin flush

solution at a concentration of 100 U /ml. In addition, the catheter will only need to remain open for several days at most. The Broviac can be studied at 24-48 hours after cell reinfusion to provide information about the rate of early thrombosis of the portal venous system. Such data will have to be collected to ultimately answer questions about the efficacy of this approach. Our experience with the baboon indicates this may not be a problem.

Infection of the catheter may also occur. In any patient who may require a liver transplant, the threat of infection is serious. The development of infection may cause the transplant to be deferred for a period of time with an obvious negative impact on patient well being. These patients will not be given prophylactic antibiotics to minimize the risk of an adverse drug reaction. The operation will be performed in an operating room with standard sterile technique. The catheter will only need to be in place for 3-4 days, and will be inspected several times daily to watch for early signs of infection. If cellulitis or purulent drainage develop, appropriate cultures will be taken and empiric intravenous antibiotics will be started. If necessary, the catheter can be removed prior to the cell infusion. The ability to institute prompt treatment and completely remove the prosthetic device will minimize any adverse impact on the patient.

Portal vein thrombosis is the most morbid potential complication of the intraportal administration of autologous hepatocytes. There are no data on the incidence of portal vein thrombosis after the intraportal administration of autologous hepatocytes, but when pancreatic islet preps are used, the incidence is about 4% (109). The relevance of this experience to the infusion of autologous hepatocytes is discussed above. Complete thrombosis with organized clot adherent to the endothelium of the portal vein may preclude subsequent liver transplantation. In addition, portal hypertension with the development of ascites or variceal hemorrhage may cause clinical deterioration in these patients with diminished cardiovascular reserves. This form of treatment in the face of even a single case of post-infusion portal vein thrombosis would require a re-evaluation of the route of delivery. Treatment of the complications of portal hypertension are relatively standard and are used frequently in the care of patients seen at the University of Michigan.

G. Evaluation and Consequences of Engraftment

The efficacy of the treatment will be assessed through a careful analysis of associated changes in lipoprotein metabolism. In addition, liver tissue will be obtained by percutaneous biopsy 3 months after the treatment and will be subjected to various molecular studies to characterize the presence and abundance of recombinant derived RNA and DNA.

1. Metabolic Consequences of Gene Transfer

A variety of animal and human studies indicate that steady state levels of lipoproteins such as LDL are affected rather dramatically by modest changes in residual LDL receptor function of FH homozygotes. We will, therefore, assess the functional consequences of gene therapy by comparing baseline metabolic parameters prior to and following the therapy. We have carefully considered the usefulness of measuring the effects of gene therapy on lipoprotein kinetics in the patient. We have decided not to pursue these more complicated measurements in the initial studies for several reasons. Fractional catabolic rates of lipoproteins such as LDL would likely be a less sensitive indication of LDL receptor expression *in vivo* than steady state lipoprotein levels. Furthermore, kinetic studies pre- and post-treatment would represent a major inconvenience to the patient because it would involve the injection of radioactivity and would protract the inpatient evaluations, leading to substantially more phlebotomies.

Approximately 6 weeks prior to the proposed treatment, the patient will begin an appropriate diet (AHA phase III) and will withdraw all drugs that may interfere with lipoprotein metabolism. Traditional forms of therapy to lower cholesterol such as drugs, plasmapheresis, or LDL apheresis will be withheld for 6 weeks prior to and 6 weeks after the treatment.

Baseline metabolic parameters will be determined by a review of the hospital record as well as the initial evaluation to be performed 4 weeks prior to the procedure (see Sect III.B.). Lipid/lipoprotein profiles (cholesterol, triglycerides, HDL-C and LDL-C) will be determined biweekly for a 3 week period between the initial evaluation and the hospital admission for the treatment. Summarized below are the analyses to be performed during the hospitalization.

Admitting evaluation (a subset of the preliminary evaluation described in Section III.B.)
- History and physical exam
- General laboratory studies:
- CBC, platelets
- Erythrocyte sedimentation rate (ESR)
- Complete urinalysis
- Test panel II
- Test panel IV

- Assess liver function; see above

- Assess coagulation status:
- Prothrombin time
- Partial thromboplastin time
- Assess lipid/lipoprotein status:
- Fasting cholesterol, triglycerides, HDL-C, LDL-C
- Lipoprotein electrophoresis
- LPP fractionation
- C-HDL & TG in LDL, IDL, VLDL, HDL, and chylos

- Assess other atherogenic potentials:
- Fibrinogen
- PAI
- LP(a)

- Cardiac evaluation:
- Chest X-ray
- EKG
- Cardiology consult

Evaluation for 7 days prior to liver resection.
- Daily fasting cholesterol, triglycerides, HDL-C, LDL-C

Evaluation for 10 days after liver resection
- Daily fasting cholesterol, triglycerides, HDL-C, LDL-C
- Daily EKG
- Every other day liver function tests
- Every other day CBC

Evaluation following discharge from hospital.

- Fasting cholesterol, triglycerides, HDL-C, LDL-C to be performed
  Biweekly- first month

Weekly- month to month 6
PRN (at least monthly)- month 6 and beyond

Liver biopsy -month 3

Complete metabolic screen as described in the preliminary evaluation annually

2. Molecular Analysis of Liver Biopsy

Techniques for detecting gene transfer and recombinant gene expression in liver tissue from recipients of autologous, retroviral transduced hepatocytes, have been developed. Examples of the application of these technologies in the analysis of the WHHL experiments are summarized in section II.C. and presented in reprints contained in the Appendix section. The major problem with the analysis of the percutaneous biopsy specimen will be the limited amount of material that will be available. Based on our extensive experience in the WHHL rabbit we believe we can do the following analyses with the material provided.

- *In situ* hybridization to detect RNA from fresh frozen tissue (See Ref. 16, 18)
- Immunocytochemistry to detect LDL receptor protein from fresh frozen tissues (Under development).
- Routine histology from paraffin sections
-Polymerase chain reaction to detect proviral DNA (See Ref. 16)
-Polymerase chain reaction to detect proviral RNA (Routine in the laboratory)

Additional assays are available if more liver tissue becomes available such as RNase protection analysis (16 and 18) or Western blot analysis (Ref. 18).

The specific protocol will be as follows: two-thirds of the biopsy will be fresh frozen in OCT and cyrostat sections will be postfixed in paraformaldehyde for *in situ* hybridization and also fixed in acetone for immunocytochemistry. The remaining tissue will be divided in half and frozen for subsequent PCR studies or fixed in paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

H. Evaluation of Rejection

1. Identification of Possible Immunologic Consequences of Autologous Cell Transfer

This proposal involves the harvest, transformation and reimplantation of autologous hepatocytes. This raises several concerns about the potential immune response of the recipient to these cells. The most favorable aspect of this protocol is the use of autologous hepatocytes for cell transfer. Because these are autologous cells, there is no risk of rejection on the basis of histo-incompatibility and no immunosuppressive therapy will be used. This markedly decreases the potential risks for the patients and also will reduce the possibility that inflammation from rejection episodes might damage the recipient's liver or spleen. It also makes it unlikely that the patients would develop cytotoxic HLA antibodies that could prevent a subsequent conventional therapy with an orthotopic liver transplant. Therefore, this protocol offers less immunologic risk to the patient than conventional liver transplantation and will not decrease the likelihood that patients could subsequently receive an orthotopic liver transplant, if necessary, to control their hypercholesterolemia.

In contrast to the low risk of HLA sensitization, reasonable concerns about immune responses to the transferred cells focus on the introduction of new and unique proteins into an

immunocompetent recipient. The recombinant derived LDL receptor protein may be new to the recipient, and therefore self-tolerance to these proteins may not have developed. This opens the possibility that the recipient could make an immune response to these new proteins that could destroy the transplanted cells. In addition, changes in the transfected cells, such as inappropriate expression of Class II HLA antigens, could cause the recipients to develop an immune response to hepatocyte proteins in a manner similar to autoimmunity.

Arguing against these latter two consequences are the results seen in autologous cell transfers in rabbits, where no immune response to the LDL receptor was noted. However, in other systems, such as the transfection of human growth hormone genes with autologous rat fibroblasts (113), immune responses to the transfected protein have occurred. Because of this example and the differences between the human and rabbit immune system, the possibility of an immune response to neoantigens on the transferred hepatocytes remains an important concern and immune responses to the LDL receptor, and the transduced hepatocytes will have to be monitored throughout the course of the cell transplant.

2. Monitoring and Detection of Immune Responses to Transduced Cell Proteins

Immune responses to several different types of antigens will be monitored throughout the course of the protocol.

Serologic Assays. Antibodies to the LDL receptor will be measured by two different techniques. Initial serologic reactivity to the LDL receptor will be assayed by Western blots of FH fibroblasts transfected with the LDL receptor and by immunoprecipitation in *in vitro* translated whole LDL receptor. Presence of glycosylated epitopes will be determined by evaluating Western blot reactivity to membranes treated with periodate. Localization of antibody epitopes in the LDL receptor will be performed by immunoprecipitation of carboxyl terminal truncations of the receptor produced by *in vitro* translation or by reactivity with fragments of the LDL receptor protein produced in a bacterial expression system. Due to the relative insensitivity of the Western blot assay, a dual capture ELISA assay for antibodies to LDL receptor also will be employed. This assay uses a mouse monoclonal antibody(115,116) to bind the LDL receptor onto the wells of an ELISA plate. After the non-specific proteins are washed off, human sera is added and specific antibodies will bind to the immobilized LDL receptor. The bound antibody will then by detected with an enzyme conjugated second antibody. This assay is more sensitive than the Western blot assays but shows slightly less specificity. In consideration of this, both LDL receptor-antibody assays will be performed. These studies will be done, pre-transplant and at one week intervals post transplant for approximately 90 days.

Many of these serologic techniques were developed and have been employed to measure immune responses in gene transfer experiments in the WHHL rabbit model of FH. Because conformation and glycosylation determinants of the LDL receptor may be involved as epitopes in an antibody response, it was decided to develop assays that use whole LDL receptor preparations produced in eukaryotic cells. Fibroblasts from FH patients (lacking a native LDL receptor) were transfected with the rabbit LDL receptor and grown in culture. Membrane preparations made from these cells were then electrophoresed in SDS-PAGE under non-reducing conditions, and transferred to nitrocellulose. The transfers were then probed with sera from WHHL rabbits that had received a transplant of autologous hepatocytes transfected with rabbit LDL receptor. Control blots were performed in parallel using fibroblasts transfected with a control vector. Results of these studies suggested that rabbits having long-term decreases in serum lipid levels did not develop serologic responses to the receptor. Additional studies are being conducted to localize serologic determinants in the receptor. To do this, the receptor has been place in a Bluescript II vector for use in immunoprecipitation studies. This plasmid will be cut at various locations throughout the LDL receptor coding region, transcribed *in vitro*, and

translated to produce carboxyl terminal truncation products that will be used in immunoprecipitation studies. In addition, two monoclonal antibodies (4D4 and 9H9) recognizing different portions of the LDL receptor have been obtained and ascites produced to serve as positive controls for these assays.

It is possible, albeit unlikely, that the antibody response to the transplanted cells will be sufficiently intense to cause a systemic reaction or serum-sickness type reaction. This would be initially evaluated through the detection of anti-LDL receptor antibodies, however, to confirm that the transplant was responsible for the reaction, further studies would be performed. Circulating immune complexes would be isolated from serum through differential polyethyleneglycol precipitation. These complexes can then be probed for specific antigens (LDL receptor) related to the transplant using Western blot analysis and anti-LDL receptor monoclonal antibodies.

Cellular Studies. Transplant recipients will be analyzed for the development of T cell immune responses to the transplanted cells.

The requirements for an antigen to use in studies identifying T cell immune responses to the LDL receptor are very different from those needed for serologic studies. Glycosylation and conformation are not important, however, removal of substances capable of causing non-specific lymphocyte activation (such as endotoxin) is essential. One method is to employ autologous cells for antigen presentation and as targets of cellular cytotoxicity. However, it would be technically easier to have isolated fragments of the receptor to use as antigen-specific stimulants in PBMC cultures. With the recent cloning of LDL receptor fragments into the bacterial expression vector, pMALc RI, and the ability of this unique fusion protein produced by it, receptor fragments can be isolated from contaminating bacterial proteins and lipopolysaccarides can be used in lymphocyte proliferation studies.

LDL receptor stimulations of PBMC will be performed using several concentrations of the recombinant proteins produced in the pMALc RI expression system. T cell responses will be determined by measuring ^{3}H Thymidine incorporation in the PBMC. Specificity of the immune response for mutational areas of the LDL receptor will be determined by comparing T cell responses to whole native versus normal LDL receptor. In addition, synthetic peptides corresponding to the regions of the mutations in the LDL receptor will be used to stimulate the PBMC to determine if the T cell responses to receptor are specific for these areas. Cytotoxic cellular immune responses will be determined by transfecting autologous cell lines (lymphoblastoid or fibroblasts) from the subject with the LDL receptor. These cells would then be ^{51}Cr labeled and used as targets in a chromium release assay.

Through these methods it is hoped that any form of immune response to the transplanted hepatocytes would be identified and characterized during the course of the experiment and the results of such an immune response could be correlated with outcome parameters to determine if it effected the transplanted cells. It may also be important to correlate observed immune responses to the LDL receptor defect phenotype of the recipient. This would clarify whether receptor negative individuals with gene deletions or nonsense mutations are more likely to develop immune reactions to transplanted proteins than recipients who have defective, but immunologically intact endogenous proteins.

IV. Overall Assessment of Risks vs. Benefits

A. Potential Efficacy of Gene therapy

Critical to an assessment of the potential efficacy of this procedure is an estimate of the expected level of genetic reconstitution. We demonstrated in preliminary studies of cultured human hepatocytes, retrovirus mediated gene transfer into a large proportion of cells (Southern blot measured 0.1 to 1.0 proviral copies/cell; Ref. 19). The retroviral vectors express levels of recombinant LDL receptor protein that exceeds normal endogenous levels by at least 5 fold. The net level of LDL receptor function in the transduced population of FH hepatocytes should be between 50% to 500% of the level of endogenous receptor assuming efficiencies of gene transfer described above. We propose to transplant approximately 5×10^9 hepatocytes or 2% of the total number of hepatocytes that are in an intact liver (approximately 2.5×10^{11} hepatocytes). We, therefore, estimate that the level of genetic reconstitution should be between 1 and 10% of endogenous LDL receptor function in FH recipients.

Several important questions emerge regarding the feasibility and efficacy of this form of therapy. The first question relates to the feasibility of obtaining this level of genetic reconstitution for prolonged periods of time using *ex vivo* gene therapy. This assumes that 1) the infused hepatocytes efficiently engraft and persist, and 2) the hepatocytes continue to express the recombinant gene and retain the differentiated functions necessary to affect improved hypercholesterolemia. Our experiments in the WHHL rabbit, described in section II.C.1 and in Appendix G, indicate that the hypothesis and assumptions noted above are valid and that the human experiments are feasible (18). Using a similar protocol to that proposed for the human experiments, we have transplanted 2×10^8 autologous, genetically-corrected hepatocytes (approximately 2% of the total hepatocytes in the liver of a rabbit) into 3 kg WHHL rabbits and have achieved genetic reconstitution in liver, as measured by quantitative RNase protection analysis, equal to 2-4% of normal endogenous RNA levels. The level of recombinant derived RNA in liver did not diminish for a period of 6 months following the transplant, the end point of the experiment. The preclinical studies in the WHHL rabbit indicate that the genetically corrected hepatocytes engraft and continue to express the recombinant gene for long periods of time.

The other critical question relates to the expected therapeutic efficacy of 2-4% hepatic LDL receptor reconstitution. Several lines of evidence suggest that this level of genetic reconstitution may lead to substantial improvements in hypercholesterolemia. Indirect evidence to support this hypothesis in man was provided by studies of Sprecher *et al.*, who characterized a population of FH homozygotes with respect to serum cholesterol and residual LDL receptor activity (41). They found an inverse correlation between serum cholesterol and residual LDL receptor activity in cultured fibroblasts. More direct support for this hypothesis has been provided in a series of hepatocyte transplantation experiments in the WHHL rabbit. Two groups have demonstrated substantial declines in serum cholesterol (25-40% decrease) in WHHL rabbits transplanted with limited numbers of hepatocytes (2-5% of the total number of hepatocytes in a liver) derived from an LDL receptor expressing allogeneic donor (17, 89). We have shown similar improvements in serum cholesterol (decreases of 25-40%) in WHHL rabbits transplanted with allogeneic or autologous hepatocytes (representing 2% of the total number of hepatocytes in a liver) that were genetically corrected *in vivo* with recombinant retroviruses (16, 18). The level of genetic reconstitution obtained in the WHHL gene therapy experiments was confirmed to be approximately 4% of endogenous hepatic levels using a quantitative RNase protection assay.

The final question, in terms of efficacy, is the potential benefit of a persistent but incomplete correction of hypercholesterolemia in improving the morbidity and mortality due to CAD. There is a huge volume of epidemiologic data that supports a relationship between LDL cholesterol and the development of CAD in the general population (117-120). The disease FH also confirms this concept in that homozygotes with LDL-cholesterol levels in the 500 to 1000 mg/dl range have much worst disease than heterozygotes with LDL-cholesterol levels in the 300 mg/dl range (2). It seems logical to assume that a 33% decline in LDL-cholesterol from 800 mg/dl to 560 mg/dl, for example, may be therapeutic. Genetic heterogeneity in the FH population has provided some insight into the possible relationships between modest differences in serum cholesterol, such as that expected from gene therapy, and the natural history of the disease. Brewer and colleagues have examined this relationship in a series of studies performed at the NIH. They showed a negative correlation between residual LDL receptor activity (2-29% of normal) and serum LDL-cholesterol (838-304 mg/dl) and a positive correlation between LDL receptor activity and age of onset of angina (41). A separate study categorized these patients with respect to presence or absence of CAD (43). The CAD symptomatic group had substantially greater LDL cholesterols (817 +/- 62 mg/dl) than the asymptomatic group (561 +/- mg/dl). Four of seven patients died in the symptomatic group during the course of the study.

B. Risks of Hepatic Resection and Hepatocyte Infusion

Resection of the left lateral segment of the liver is a fairly straightforward surgical procedure that is associated with some risk. The liver resection should be accomplished without the necessity of a transfusion. Mortality as a result of this procedure should be low; the most likely cause of mortality would be a cardiac event occurring in the perioperative period. The incidence of this should be approximately 1% (95,96). The patient will undergo extensive preoperative evaluation and intraoperative hemodynamic monitoring to minimize these risks. Possible noncardiac morbidity could result from the liver resection and catheter placement in the portal circulation. These include 1) excessive bleeding as a result of the resection, 2) infection at the site of the hepatectomy or via the indwelling catheter, and 3) leakage of bile causing peritonitis. This procedure might make subsequent orthotopic liver transplantation more difficult but it wouldn't preclude a successful organ transplant. Removal of the catheter should be associated with little morbidity. Removal will involve no further anesthetic, and can be done at the bedside.

Infusion of the hepatocytes via the indwelling Broviac catheter into the portal circulation is also associated with risks. The most morbid complication of this procedure is portal vein thrombosis. There are no data in humans on the incidence of portal vein thrombosis after the intraportal administration of autologous hepatocytes, but when pancreatic islet preps are used, the incidence was about 4% (109). We expect a lower incidence of portal vein thrombosis with autologous hepatocytes because the cells are autologous rather than allogeneic and the infusate contains a suspension of disaggregated cells rather than tissue fragments. Complete thrombosis with organized clot adherent to the endothelium of the portal vein may preclude subsequent liver transplantation. In addition, the sequalae of portal vein thrombosis, portal hypertension with the development of ascites or variceal hemorrhage may cause clinical deterioration in these patients with diminished cardiovascular reserves. The studies done in the WHHL rabbit have not shown portal vein thrombosis to date. Furthermore, our experience in the baboon model indicates that portal vein thrombosis or portal vein hypertension may not be a problem. Treatment of the complications of portal hypertension are relatively standard. Additional complications of the hepatocyte infusion are 1) anaphylaxis, and 2) infection of the Broviac catheter. Removal will involve no further anesthetic, and can be done at the bedside. It is a common procedure.

C. Risks of Gene Transfer and Gene Expression

The only possible complication of LDL receptor gene transfer per se would be an immune response to the recombinant derived LDL receptor protein. Issues that relate to this potential problem have been described under Section III.H. It is difficult to predict the likelihood of this occurring, however, our work in the "receptor negative" WHHL rabbit (Appendix G) suggests that immunologic rejection may not be a confounding or limiting problem. The most morbid consequence of an immune response to the recombinant derived receptor would simply be loss of functional engraftment.

Risks of gene transfer using retroviral vectors have been addressed extensively in animal models and more recently in human subjects (Reviewed in Ref. 121). Possible complications include 1) the development of replication competent virus and sequalae of viral replication *in vivo*, and 2) long-term consequences of random retroviral integration such as the development of secondary malignancies.

A significant effort has been made to minimize the risks of formation of replication competent viruses. The cell line that produces the virus has been engineered to prevent the formation of wild-type virus (See Section C.). The potential toxicity of inadvertent formation of replication competent murine retroviruses has been addressed in a series of experiments performed at the NIH (121, 122). Four nonhuman primates were infused with large volumes of replication competent amphotropic virus. The virus was rapidly cleared and the animals remained free of disease up to the time of their report (mean follow-up was 43.9 months). Additional experiments in which monkeys were immunosuppressed and transplanted with virus producing autologous fibroblasts were associated with no ontoward effects for the average follow-up of 61.8 months. Based on these extensive studies we conclude that the formation of replication competent virus is highly unlikely and if it occurred would probably not cause disease.

A more difficult safety issue to address is the risk of malignant transformation due to retroviral mediated gene transfer. The theoretical basis for this has been nicely reviewed by Cornetta et al. (121) The major concern relates to the fact that retroviruses insert into the recipient cell's genome in a near-random manner and that this insertion event could potentially lead to the activation of an oncogene or the inactivation of a tumor suppressor gene. While this is theoretically possible the chance of a secondary malignancy is highly unlikely for several reasons. Transformation of human cells *in vivo* is clearly a multistep process and a single event, such as insertion of a provirus, will probably not lead to the formation of a *de novo* tumor. Furthermore, insertional inactivation of a tumor suppressor gene would be insufficient for transformation because one allele would remain intact and capable of providing tumor suppression function. While the insertion may not itself cause transformation it may bring the cell one step closer to transformation. Applications of retrovirus mediated gene transfer for treatment of humans must take into account this potential tumor promoting capability. We believe this will not be a major problem in our protocol for several reasons. We will be working with a population of patients that are relatively young and have a short life expectancy. Development of secondary malignancies long after the therapy will not be an issue. Furthermore the patient's tumor surveillance systems will remain intact because they will not be immunosuppressed.

Another aspect of our protocol that makes transformation highly unlikely relates to the nature of the recipient cell for gene transfer, the hepatocyte. Under normal conditions, the hepatocyte is a terminally differentiated cell which is capable of undergoing very limited number of cell divisions *in vivo* or *in vitro*. It is extremely difficult to immortalize or

transform cultured hepatocytes even with known oncogenic DNA viruses such a adenoviruses or SV40.

Additional support for the safety of transplanting transduced hepatocytes is provided by our experiments in animals. We have performed *ex vivo* gene therapy with retroviral transduced hepatocytes into at least 40 rabbits and 25 rats. The animals have been followed up to 6 months. During this time we have not observed any tumors or malignancies.

D. Summary of Risks and Benefits

The potential benefits of this procedure to FH patients will be to slow the progression of atherosclerosis and decrease morbidity and mortality of CAD. Gene therapy would be performed in a group of FH patients who have developed advanced CAD on traditional therapies and have a relatively poor prognosis. Experiments in an authentic animal model for FH have demonstrated the feasibility of achieving substantial and prolonged diminutions of total serum cholesterol as a result of gene therapy . We do not expect the improvement in hypercholesterolemia to be complete so that we consider this therapy an adjunct to more standard therapies such as plasmapheresis, LDL-apheresis, and/or pharmacologic agents. Patients that receive gene therapy would resume their normal therapeutic programs 6 weeks later. The development of gene therapies is necessary because the currently available forms of treatment are inadequate. Most traditional forms of therapy lead to only partial improvements in hypercholesterolemia and CAD usually progresses unabated despite intensive therapeutic regimens.

The potential risks of this therapy are complex. The risks of the surgical procedures are relatively well defined and are based on extensive experience in similar clinical situations. The hepatocyte infusion is likely to be the most morbid procedure although it is difficult to predict these risks because infusion of autologous hepatocytes into humans has never been done. Our early experience in nonhuman primates is encouraging. Experience with islet cell transplantation in humans suggests that the risk of portal vein thrombosis is less than 4%. The risks associated with gene transfer and random integration into the genome are largely unknown but are expected to be quite low.

V. References

1. Brown MS and Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science, Apr 4 1986;232(4746):34-47.
2. Goldstein JL and Brown MS. Familial hypercholesterolemia. Lipoprotein and lipid Metabolism Disorders. Metabolic Basis of Inherited Disease II 1989;1:1215-1250.
3. Bilheimer DW. Portacaval shunt and liver transplantation in treatment of familial hypercholesterolemia. Arteriosclerosis, Jan-Feb 1989;9(1 Suppl):I158-63.
4. Thompson GR, Barbir M, Okabayashi K, Trayner I and Larkin S. Plasmapheresis in familial hypercholesterolemia. Arteriosclerosis, Jan-Feb 1989;9(1 Suppl):I152-57.
5. Starzl TE, Bilheimer DW, Bahnson HT, Shaw BW Jr, Hardesty RL, Griffity BP, Iwatsuki S, Zitelli BJ, Gartner JC Jr, Malatack, JJ and et al. Heart-liver transplantation in a patient with familial hypercholesterolemia. Lancet, Jun 23 1984;1(8391):1382-83.
6. Bilheimer DW, Goldstein JL, Grundy SM, Starzl TE and Brown MS. Liver transplantation to provide low density lipoprotein receptors and lower plasma cholesterol in a child with homozygous familial hypercholesterolemia. N Engl J Med, Dec 27 1984;311(26):1658-64.
7. Shaw BW Jr, Bahnson HT, Hardesty RL, Griffity BP and Starzl TE. Combined transplantation of the heart and liver. Ann Surg, Dec 1985;202(6):667-72.
8. Hoeg JM, Starzl TE and Brewer HB Jr. Liver transplantation for treatment of cardiovascular disease: comparison with medication and plasma exchange in homozygous familial hypercholesterolemia. Am J Cardio, Mar 1 1987;59(6):705-07.
9. Valdivielso P, Escolar JL, Cuervas-Mons V, Pulpon LA, Chaparro MA and Gonzalez-Santos P. Lipids and lipoprotein changes after heart and liver transplantation in a patient with homozygous familial hypercholesterolemia. Ann Intern Med, Feb 1988;108(2):204-06.
10. Watanabe Y. Serial inbreeding of rabbits with hereditary hyperlipidemia (WHHL rabbit). Atherosclerosis, Jun 1980;36(2):261-8.
11. Goldstein JL, Kita T and Brown MS. Defective lipoprotein receptors and atherosclerosis. Lessons from an animal counterpart of familial hypercholesterolemia. N Engl J Med, Aug 4 1983;309(5):288-96.
12. Yamamoto T, Bishop RW, Brown MS, Goldstein JL and Russell DW. Deletion in cysteine-rich region of LDL receptor impedes transport to cell surface in WHHL rabbit. Science, Jun 6 1986;232(4755):1230-37.
13. Havel RJ, Yamada N and Shames DM. Watanabe heritable hyperlipidemic rabbit. Animal model for familial hypercholesterolemia. Arteriosclerosis, Jan-Feb 1989;9(1 Suppl):I33-38.
14. Wilson JM, Johnston DE, Jefferson DM and Mulligan RC. Correction of the genetic defect in hepatocytes from the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci USA, Jun 1988;85(12):4421-25.
15. Wilson, JM and Chowdhury JR. Prospects for gene therapy of familial hypercholesterolemia. Mol Biol Med, Jun 1990;7(3):223-32.
16. Wilson JM, Chowdhury NR, Grossman M, Wajsman R, Epstein A, Mulligan RC and Chowdhury JR. Temporary amelioration of hyperlipidemia in low density lipoprotein receptor-deficient rabbits transplanted with genetically modified hepatocytes. Proc Natl Acad Sci USA, Nov 1987;87(21):8437-41.
17. Wilson JM, Chowdhury NR, Grossman M, Gupta SJ, Jeffers J, Huang TJ and Chowdhury JR. Transplantation of allogeneic hepatocytes into LDL receptor deficient rabbits leads to transient improvement in hypercholesterolemia. Clin Biotechnology, Spring 1991. (In Press).

18. Chowdhury JR, Grossman M, Gupta SJ, Chowdhury NR, Baker JR Jr and Wilson JM. Ex vivo gene therapy in LDL receptor deficient rabbits leads to permanent improvement of hypercholesterolemia. (Submitted).
19. Grossman M, Raper S and Wilson JM. Towards liver-directed gene therapy: retrovirus mediated gene transfer into human hepatocytes. (Submitted).
20. Friedmann T. Progress toward human gene therapy. Science, Jun 16 1989;244(4910):1275-81.
21. Wilson JM, Jefferson DM, Chowdhury JR, Novikoff PM, Johnston DE and Mulligan RC. Retrovirus-mediated transduction of adult hepatocytes. Proc Natl Acad Sci USA, May 1988;85(9):3014-18.
22. Williams DA, Lemischka IR, Nathan DG and Mulligan RC. Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature, Aug 9-15 1984;310 (5977):476-80.
23. Rosenfeld MA, Siegfried W, Yoshimura K, Yoneyama K, Fukayama M, Stier LE, Paakko PK, Gilardi P, Stratford-Perricaudet LD, Perricaudet M, Jalat S, Pavirani A, Lecocq JP and Crystal RG. Adenovirus-mediated transfer of a recombinant α1-antitrypsin gene to the lung epithelium in vivo. Science, Apr 19 1991;252:431-34.
24. Culver K, Cornetta K, Morgan R, Morecki S, Aebersold P, Kasid A, Lotze M, Rosenberg SA, Anderson WF and Blaese RM. Lymphocytes as cellular vehicles for gene therapy in mouse and man. Proc Natl Acad Sci USA, Apr 15 1991;88(8):3155-59.
25. Wilson JM, Birinyi LK, Salomon RN, Libby P, Callow AD and Mulligan RC. Implantation of vascular grafts lined with genetically modified endothelial cells. Science, Jun 16 1989;244(4910):1344-46.
26. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A and Felgner PL. Direct gene transfer into mouse muscle in vivo. Science, Mar 23 1990;247(4949 Pt 1):1465-68.
27. Lin H, Parmacek HS, Morle G, Bolling S and Leiden JM. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation, Dec 1990;82(6):2217-21.
28. Axelrod JH, Read MS, Brinkhous KM and Verma IM. Phenotypic correction of factor IX deficiency in skin fibroblasts of hemophilic dogs. Proc Natl Acad Sci USA, July 1990;87(13):5173-77.
29. Morgan JR, Barrandon Y, Green H and Mulligan RC. Expression of an exogenous growth hormone gene by transplantable human epidermal cells. Science, Sep 18 1987;237(4821):1476-79.
30. Palella TD, Hidaka Y, Silverman LJ, Levine M, Glorioso J and Kelley WN. Expression of human HPRT mRNA in brains of mice infected with a recombinant herpes simplex virus-1 vector. Gene, Aug 1 1989;80(1):137-44.
31. Cepko CL, Roberts BE and Mulligan RC. Construction and applications of a highly transmissable murine retrovirus shuttle vector. Cell, Jul 1984;37(3):1053-62.
32. Samulski RJ, Chang LS and Shenk T. Helper-free stocks of recombinant adeno-associated viruses: Normal integration does not require viral gene expression. J Virol, Sep 1989;63(9):3822-28.
33. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM and Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA, Nov 1987;84(21):7413-17.
34. Nicolau C and Legrand A, Grosse E. Liposomes as carriers for in vivo gene transfer and expression. Methods Enzymol, 1987;149:157-76.
35. Wu GY and Wu CH. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system [published erratum appears in J Biol Chem, Jan 5, 1988;263(1):588], J Biol Chem, Apr 5 1987;262(10):4429-32.
36. Russell DW, Esser V and Hobbs HH. Molecular basis of familial hypercholesterolemia. Arteriosclerosis, Jan-Feb 1989;9(1 Suppl):I8-13.

37. Lodstein JL, Dana SE and Brown MS. Esterification of low density lipoprotein cholesterol in human fibroblasts and its absence in homozygous familial hypercholesterolemia. Proc Natl Acad Sci USA, Nov 1974;71(11):4288-92.
38. Glickman RM and Sabesin SM. Lipoprotein metabolism. In The Liver: Biology and Pathophysiology (Aria IM, Jakoby WB, Popper H, Schachter D and Shafritz DA, eds), 2nd edit., 1988, pp. 331-354, Raven Press Ltd, New York.
39. Turley SD and Dietschy JM. The metabolism and excretion of cholesterol by the liver. In The Liver: Biology and Pathophysiology (Aria IM, Jakoby WB, Popper H, Schachter D and Shafritz DA, eds), 2nd edit., 1988, pp. 617-641, Raven Press Ltd, New York.
40. Robins SJ, Fasulo JM, Collins MA and Patton GM. Evidence for separate pathways of transport of newly synthesized and preformed cholesterol into bile. J Biol Chem, Jun 10 1985;260(11):6511-3.
41. Sprecher DL, Hoeg JM, Schaefer EJ, Zech LA, Gregg RE, Lakatos E and Brewer HB, Jr. The association of LDL receptor activity, LDL cholesterol level, and clinical course in homozygous familial hypercholesterolemia. Metabolism, Mar 1985;34(3):294-99.
42. Goldstein JL and Brown MS. The LDL receptor defect in familial hypercholesterolemia. Implications for pathogenesis and therapy. Med Clin North Am, Mar 1982;66(2):335-62.
43. Sprecher DL, Schaefer EJ, Kent KM, Gregg RE, Zech LA, Hoeg JM, McManus B, Roberts WC and Brewer HB, Jr. Cardiovascular features of homozygous familial hypercholesterolemia analysis of 16 patients. Am J Cardiol, July 1 1984;54(1):20-30.
44. Haitas B, Baker SG, Meyer TE, Joffe BI and Seftel HC. Natural history and cardiac manifestations of homozygous familial hypercholesterolemia. Q J Med, Jul 1990;76(279):731-40.
45. Khachadurian AK and Uthman SM. Experiences with the homozygous cases of familial hypercholesterolemia. A report of 52 patients. Nutr Metab, 1973;15(1):132-40.
46. Mabuchi H, Tatami R, Haba T, Ueda K, Ueda R, Kametani T, Itoh S, Koizumi J, Oota M, Miyamoto S, Takeda R and Takeshita H. Homozygous familial hypercholesterolemia in Japan. Am J Med, Aug 1978;65(2):290-97.
47. Mabuchi H, Koizumi H, Shimizu M and Takeda R. Development of coronary heart disease in familial hypercholesterolemia. Circulation, Feb 1989;79(2):225-32.
48. Moorjani S, Roy M, Gagne C, Davignon J, Brun D, Toussaint M, Lambert M, Campeau L, Blaichman S. and Lupien P. Homozygous familial hypercholesterolemia among French Canadians in Quebec Province. Arteriosclerosis, Mar-Apr 1989;9(2):211-16.
49. Lehrman MA, Schneider WJ, Brown MS, David CG, Elhammer A, Russell DW and Goldstein JL. The Lebanese allele at the low density lipoprotein receptor that is retained in endoplasmic reticulum. J Biol Chem, Jan 5 1987;262(1):401-10.
50. Leitersdorf E., Tobin EJ, Davignon J and Hobbs HH. Common low-density lipoprotein receptor mutations in the French Canadian population. J Clin Invest, Apr 1990;85(4):1014-23.
51. Goldstein JL and Brown MS. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. J Lipid Res, Dec 15 1984;25(13):1450-61.
52. Sudhof TC, Russell DW, Brown MS and Goldstein JL. 42 bp element from LDL receptor gene confers end-product repression by sterols when inserted into viral TK promoter. Cell, Mar 27 1987;48(6):1061-69.
53. Miyanohara A, Sharkey MF, Witztum JL, Steinberg D and Freidmann T. Efficient expression of retroviral vector-transduced human low density lipoprotein (LDL) receptor-deficient rabbit fibroblasts in vitro. Proc Natl Acad Sci USA, Sep 1988;85(17):6538-42.

54. Yamamoto T, Davis CG, Brown MS, Schneider WJ, Casey ML, Goldstein JL and Russell DW. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. Cell, Nov 1984;39(1):27-38.
55. Davis CG, van Driel IR, Russell DW, Brown MS and Goldstein JL. The low density lipoprotein receptor. Identification of amino acids in cytoplasmic domain required for rapid endocytosis. J Biol Chem, Mar 25 1987;262(9):4075-82.
56. Hofmann SL, Russell DW, Brown MS, Goldstein JL and Hammer RE. Overexpression of low density lipoprotein (LDL) receptor eliminates LDL from plasma in transgenic mice. Science, Mar 11, 1988;239(4845):1277-81.
57. Yokode M, Hammer RE, Ishibashi S., Brown MS and Goldstein JL. Diet-induced hypercholesterolemia in mice: prevention by overexpression of LDL receptors. Science, Nov 30 1990;250(4985):1273-75.
58. Khachadurian AK. Cholestyramine therapy in patients homozygous for familial hypercholesterolemia (familial hypercholesterolemic xanthomatosis). J Atheroscler Res, Jan-Feb 1968;8(1):177-88.
59. Levy RI, Fredrickson DS, Stone NJ, Bilheimer DW, Brown WV, Glueck CJ, Gotto AM, Herbert PN, Kwiterovich PO, Langer T, LaTosa J, Lux SE, Rider AK, Shulman RS and Sloan HR. Cholestyramine in type II hyperlipoproteinemia. A double-blind trial. Ann Intern Med, Jul 1973;79(1):51-8.
60. Yamamoto A, Yamamura T, Yokoyama S, Sudo H and Matsuzawa Y. Combined drug therapy--cholstyramine and compactin--for familial hypercholesterolemia. Int J Clin Pharmacol Ther Toxicol, Sep 1984;22(9):493-7.
61. Malloy MJ, Kane JP, Kunitake ST and Tun P. Complementarity of colestipol, niacin, and lovastatin in treatment of severe familial hypercholesterolemia. Ann Intern Med, Nov 1987;107(5):616-23.
62. Uauy R, Vega GL, Grundy SM and Bilheimer DM. Lovastatin therapy in receptor-negative homozygous familial hypercholesterolemia: lack of effect on low-density lipoprotein concentrations or turnover. J Pediatr, Aug 1988;113(2):387-92.
63. Stein Ea. Treatment of familial hypercholesterolemia with drugs in children. Arteriosclerosis, Jan-Feb 1989;9(1 Suppl):I145-51.
64. Starzl TE, Putnam CW, Chase HP and Porter KA. Portacaval shunt in hyperlipoproteinaemia. Lancet, Oct 27 1973;2(835):940-44.
65. Starzl TE, Chase HP, Ahrens EH Jr, McNamara DJ, Bilheimer DW, Schaefer EJ, Rey J, Porter KA, Stein E, Francavilla A and Benson LN. Portacaval shunt in patients with familial hypercholesterolemia. Ann Surg, Sep 1983;198(3):273-83.
66. Thompson GR, Lowenthal R and Myant NB. Plasma exchange in the management of homozygous familial hypercholesterolemia. Lancet, May 31 1975;1(7918):1208-11.
67. Apstein CS, Zilversmit DB, Lees RS and George PK. Effect of intensive plasmapheresis on the plasma cholesterol concentration with familial hypercholesterolemia. Atherosclerosis, Oct 1978;31(2):105-15.
68. King ME, Breslow JL and Lees RS. Plasma-exchange therapy of homozygous familial hypercholesterolemia. N Engl J Med, Jun 1980;302(26):1457-9.
69. Myant NB. Regression of coronary atherosclerosis in man. Adv Exp Med Biol, 1984;168:139-52.
70. Yokoyama S, Hayashi R, Satani M and Yamamoto A. Selective removal of low density lipoprotein by plasmapheresis in familial hypercholesterolemia. Arteriosclerosis, Nov-Dec 1985;5(6):613-22.
71. Postiglione A and Thompson GR. Experience with plasma-exchange in homozygous familial hypercholesterolemia. Prog Clin Biol Res, 1985;188:213-20.
72. Thompson GR, Miller JP and Breslow JL. Improved survival of patients with homozygous familial hypercholesterolemia treated with plasma exchange. Br Med J [Clin Res], Dec 14, 1985;291(6510):1671-3.

73. Keller C, Schmitz H, Theisen K and Zollner N. Regression of valvular aortic stenosis due to homozygous familial hypercholesterolemia following plasmapheresis. Klin Wochenschr, Apr 1 1986;64(7):338-41.
74. Stein EA, Adolph R, Rice V, Glueck CJ and Spitz HB. Nonprogression of coronary artery atherosclerosis in homozygous familial hypercholesterolemia after 31 months of repetitive plasma exchange. Clin Cardiol, Mar 1986;9(3):115-9.
75. Lupien PJ, Moorjani S, Gagne C, Brun LD, Lou M and Dagenais G. Long term treatment of two familial hypercholesterolemic heterozygote patients with batch affinity chromatography (BAC). Artery, 1982;10(4):286-300.
76. Stoffel W and Demant T. Selective removal of apolipoprotein B-containing serum lipoproteins from blood plasma. Proc Natl Acad Sci USA, Jan 1981;78(1):611-5.
77. Yodoyama S, Hayashi R, Satani M and Yamamoto A. Selective removal of low density lipoprotein by plasmapheresis in familial hypercholesterolemia. Arteriosclerosis, Nov-Dec 1985;5(6):613-22.
78. Gwynn JT. Probucol, high-density lipoprotein metabolism and reverse cholesterol transport. Am J Cardio, Jul 25 1988;62(3):48B-51B.
79. Baker SG, Joffe Bi, Mendelsohn D and Seftel HC. Treatment of homozygous familial hypercholesterolemia with probucol. S Afr Med J, Jul 3 1982;62(1):7-11.
80. Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Oshima A, Yoshida H and Kawai C. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. Proc Natl Acad Sci USA, Aug 1987;84(16):5928-31.
81. Leonard JV, Clarke M, McCartney FJ and Slack. Progression of atheroma in homozygous familial hypercholesterolemia during regular plasma exchange [letter]. Lancet, Oct 10 1981;2(850):811.
82. Deckelbaum RJ, Lees RS, Small DM, Hedberg SE, Grundy SM. Failure of complete bile diversion and oral bile acid therapy in the treatment of homozygous familial hypercholesterolemia. N Engl J Med, Mar 3 1977;296(9):465-70.
83. Schneider WJ, Brown MS, and Goldstein JL. Kinetic defects in the processing of the low density lipoprotein receptor in fibroblasts from WHHL rabbits and a family with familial hypercholesterolemia. Mol Biol Med, Oct 1983;1(3):353-67.
84. Tanzawa K, Shimada Y, Kuroda M, Tsujita Y, Arai M, and Watanabe H. WHHL rabbit: a low density lipoprotein receptor-deficient animal model for familial hypercholesterolemia. FEBS, Aug 25 1980;118(1):81-4.
85. Pittman RC, Carew TE, Attie AD, Witzman JL, Watanabe Y, and Steinberg D. Receptor-dependent and receptor-independent degradation of low density lipoprotein in normal rabbits and in receptor-deficient mutant rabbits. J Biol Chem, Jul 1982;257(14):7994-8000.
86. Kita T, Brown MS, Watanabe Y and Goldstein JL. Deficiency of low density lipoprotein receptors in liver and adrenal gland of the WHHL rabbit, an animal model of familial hypercholesterolemia. Proc Natl Acad Sci USA, Apr 1981;78(4):2268-72.
87. Bilheimer DW, Watanabe Y and Kita T. Impaired receptor-mediated catabolism of low density lipoprotein in the WHHL rabbit, an animal model of familial hypercholesterolemia. Proc Natl Acad Sci USA, May 1982;79(10):3305-9.
88. Kita T, Brown MS, Bilheimer DW and Goldstein JL. Delayed clearance of very low density and intermediate density lipoproteins with enhanced conversion to low density lipoproteins in WHHL rabbits. Proc Natl Acad USA, Sep 1979;79(18):5693-7.
89. Wiederkehr JC, Kondos GT and Pollak R. Hepatocyte transplantation for the low-density lipoprotein receptor-deficient state. A study in the Watanabe rabbit. Transplantation, Sep 1990;50(3):466-71.
90. Danos O and Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. Proc Natl Acad Sci USA, Sep 1988;85(17):6460-4.

91. Price J, Turner D and Cepko C. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. Proc Natl Acad Sci USA, Jan 1987;84(1):156-60.
92. Godbout R, Ingram RS and Tilghman SM. Fine-structure mapping of the three mouse alpha-fetoprotein gene enhancers. Mol Cell Biol, Mar 1988;8(3):1169-78.
93. Wilson JM, Jefferson DM, Chowdhury JR, Novikoff PM, Johnston DE and Mulligan RC. Retrovirus-mediated transduction of adult hepatocytes. Proc Natl Acad Sci USA, May 1988;85(9):3014-8.
94. Yu SF, von Ruden T, Kantoff PW, Garber C, Seiberg M, Ruther U, Anderson WF, Wagner EF and Gilboa E. Self-inactivating retroviral vectors designed for transfer of whole genes into mammalian cells. Proc Natl Acad Sci USA, May 1986;83(10):3194-8.
95. Goldman L. Cardiac risks and complications of noncardiac surgery. Ann Intern Med, Apr 1983;98(4):504-13.
96. Weitz HH and Goldman L. Noncardiac surgery in the patient with heart disease. Med Clin North Am, May 1987;71(3):413-32.
97. Lim JS, Proudfit WL and Sones FM Jr. Left main coronary arterial obstruction: Long-term follow-up of 141 nonsurgical cases. Am J Cardiol, Aug 1975;36(2):131-5.
98. Bruschke AV, Proudfit WL and Sones FM Jr. Progress study of 590 consecutive nonsurgical cases of coronary disease followed 5-9 years. I. Arterographic correlations. Circulation, Jun 1973;47(6):1147-53.
99. Kramer JR, Kitazume H, Proudfit WL, Matsuda Y, Williams GW and Sones FM Jr. Progression and regression of coronary atherosclerosis:relation to risk factors. Am Heart J, Jan 1983;105(1):134-44.
100. Killip T, Passamani E and Davis K. Coronary artery surgery study (CASS) : a randomized trial of coronary bypass surgery. Eight years follow-up and survival in patients with reduced ejection fraction. Circulation, Dec 1985;72(6 Pt 2):V102-9.
101. Glueck CJ. Role of risk factor management in progression and regression of coronary and femoral artery atherosclerosis. Am J Cardiol, May 1986;57(14):35G-41G.
102. Leffert HL, Koch KS, Moran T and Williams M. Liver cells. Methods Enzymol, 1979;58:536-44.
103. Enat R, Jefferson DM, Ruiz-Opazo N, Gatmaitan Z, Leinwald LA and Reid LM. Hepatocyte proliferation *in vitro:* its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. Proc Natl Acad Sci USA, 1984;81:1411-15.
104. Korman AJ, Frantz JD, Strominger JL and Mulligan RC. Expression of human class II major histocompatibility complex antigens using retrovirus vectors. Proc Natl Acad Sci USA, Apr 1987;84(8):2150-4.
105. Van Beveren F, Coffin J and Hughes S. In RNA Tumor Viruses (Weiss R, Teich N, Varmus H and Coffin J, eds.) 2nd edit, 1985 , Cold Spring Harbor Lab., Cold Spring Harbor, NY; pp. 766-783.
106. Kost TA, Theodorakis N and Hughes SH. The nucleotide sequence of the chick cytoplasmic beta-actin gene. Nucleic Acids Res, Dec 1983;11(23):8287-301.
107. Seed B and Aruffo A. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. Proc Natl Acad Sci USA, May 1987;84(10):3365-9.
108. Boshart M, Weber F, Jahn G, Dorsch-Hasler K, Fleckenstein B and Schaffner W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell, Jun 1985;41(2):521-30.
109. Memsic L, Busuttil RW and Traverso LW. Bleeding esophageal varices and portal vein thrombosis after pancreatic mixed-cell autotransplantation. Surgery, 1984;95:238-242.
110. Lawrence TS, Dworzanin LN, Walker-Andrews SC, Andrews JC, TenHaken RK, Wollner IS, Lichter AS and Ensminger WD. Treatment of cancers involving the liver and porta

hepatitis with external beam irradiation and intraarterial hepatic fluorodeoxyuridine. Int J Radiation Oncology, 1991;29:555-561.
111. Mullen Y, Clare-Salzler M, Stein E and Clark W. Islet transplantation for the cure of diabetes. Pancreas, 1989;4(1):123-135.
112. Sterneck M, Ring E, Gordon R, LaBerge J, Roberts J, Asher N and Lake J. Intrahepatic portacaval shunt (IPCS): A bridge to liver transplantation in patients with refractory variceal bleeding. Gastroenterology, 1991;100:A193.
113. Chang PL, Capone JP and Brown GM. Autologous fibroblast implantation. Feasibility and potential problems in gene replacement therapy. Mol Biol Med, Dec 1990;7(6):461-70.
114. van Driel IR, Goldstein JL, Sudhof TC and Brown MS. First cysteine-rich repeat in ligand-binding domain of low density lipoprotein receptor binds Ca2+ and monoclonal antibodies, but not lipoproteins. J Biol Chem, Dec 1987;262(36):17443-9.
115. van Driel IR, Brown MS and Goldstein JL. Stoichiometric binding of low density lipoprotein (LDL) and monoclonal antibodies to LDL receptors in a solid phase assay. J Biol Chem, Jun 1989;264(16):9533-8.
116. Huettinger M, Schneider WJ, Ho YK, Goldstein JL and Brown MS. Use of monoclonal anti-receptor antibodies to probe the expression of the low density lipoprotein receptor in tissues of normal and Watanabe heritable hyperlipidemic rabbits. J Clin Invest, Sep 1984;74(3):1017-24.
117. Kannel WB. Lipids, diabetes, and coronary heart disease: insights from the Framingham Study. Am Heart J, Nov 1985;110(5):1100-7.
118. Castelli WP, Garrison RJ, Wilson PW, Abbott RD, Kalousdian S and Kannel WB. Incidence of coronary heart disease and lipoprotein cholesterol levels. The Framingham Study. JAMA, Nov 1986;256(20):2835-8.
119. Abbott RD, Wilson PW, Kannel WB and Castelli WP. High density lipoprotein cholesterol, total cholesterol screening, and myocardial infarction. The Framingham Study. Arteriosclerosis, May-Jun 1988;8(3):207-11.
120. Castelli WP. Cholesterol and lipids in the risk of coronary artery disease--the Framingham Heart Study. Can J Cardiol, Jul 1988;4 Suppl A:5A-10A.
121. Cornetta K, Morgan RA and Anderson WF. Safety issued related to retroviral-mediated gene transfer in humans. Hum Gene Ther, Spring 1991;2(1):5-14.
122. Cornetta K, Moen RC, Culver K, Morgan RA, McLachlin JR, Sturm S, Selegue J, London W, Blaese RM and Anderson WF. Amphotropic murine leukemia retrovirus is not an acute pathogen for primates. Hum Gene Ther, Spring 1990;1(1):15-30.